(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 November 2003 (13.11.2003)

PCT

(10) International Publication Number
WO 03/093311 A1

(51) International Patent Classification[7]: C07K 14/47, C12N 9/12, A61K 38/45, A61P 3/10

(21) International Application Number: PCT/NZ03/00074

(22) International Filing Date: 29 April 2003 (29.04.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/376,926 29 April 2002 (29.04.2002) US
524892 21 March 2003 (21.03.2003) NZ

(71) Applicant *(for all designated States except US)*: **PROTEMIX CORPORATION LIMITED** [NZ/NZ]; Level 4, 41 Shortland Street, 1001 AUCKLAND (NZ).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **COOPER, Garth, James, Smith** [NZ/NZ]; 2 Crummer Road, Ponsonby, 1001 AUCKLAND (NZ). **LOOMES, Kerry** [NZ/NZ]; C/- Level 4, 41 Shortland Street, 1001 Auckland (NZ).

(74) Agents: ADAMS, Matthew, D et al.; A J Park, 6th Floor Huddart Parker Building, PO Box 949, 6015 Wellington (NZ).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/093311 A1

(54) Title: PROTEINS WITH DEGLYCATING ACTIVITIES AND METHODS OF USING SAME

(57) Abstract: SEQ ID Nos 1 and 2are the amino acid and nucleotide sequences of the deglycating enzyme of the invention. Variants, derivatives, homologues, analogs, fragments, salts and pharmaceutical compositions thereof are also provided that have direct or indirect deglycation activity. Deglycation occurring for example, by phosphorylation of molecules on glycoproteins or other sugar-bearing proteins. The Deglycating Enzymes of the invention are capable of degradation of low molecular weight amines, amino acids, sugar amines, glycoproteins and other sugar-bearing proteins. SEQ ID No 1, Deglycating Enzymes in general and antagonists thereof are considered in methods of providing therapy-especially for treatment of diabetes.

PROTEINS WITH DEGLYCATING ACTIVITIES AND METHODS OF USING SAME

FIELD OF THE INVENTION

This invention concerns, by way of example, diabetes, hyperglycemia, and sugar-modified proteins, as well as novel proteins and therapeutic uses of such proteins believed to function as enzymes, for example as kinases, and related proteins, analogs, fragments, variants, and derivatives thereof. This invention further concerns, for example, compositions comprising such proteins, analogs, fragments, variants, and derivatives, methods of making such proteins, analogs, fragments, variants, and derivatives and related compositions, and methods of using such proteins, analogs, fragments, variants, and derivatives and related compositions, for example, to identify and assess compounds that modulate activity.

BACKGROUND OF THE INVENTION

The following description includes information that may be useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art, or relevant, to the presently described or claimed inventions, or that any publication or document that is specifically or implicitly referenced is prior art.

Diabetes mellitus is a common disorder affecting nearly 16 million Americans (Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care*, 20; 1183-97 (1997)). Diabetic individuals are prone to complications which are a major threat to both the quality and the quantity of life. Almost half those diagnosed with diabetes before the age of 31 years die before they reach 50 years largely as a result of cardiovascular or renal complications, often with many years of crippling and debilitating disease beforehand (Deckert T, Poulsen J, Larsen M. *Diabetologia* 14:363-70 (1978)).

The disease is characterised by chronic hyperglycaemia, and specific micro- and macrovascular pathologies. As a consequence, diabetes is a leading cause of blindness, end stage renal disease, myocardial infarction, limb amputation and a range of

neuropathies (Brownlee, M. *Nature* 414: 813-820 (2001)). It has been estimated that diabetic individuals have a 25-fold increase in the risk of blindness, a 20-fold increase in the risk of renal failure, a 20-fold increase in the risk of amputation as a result of gangrene, and a 2- to 6-fold increased risk of coronary heart disease and ischemic brain damage (Klein R, Klein B, Moss S, Davis M, DeMets D. *Diabetes Care* 8;311-5 (1985)).

Largely because of these long-term complications, the cost of diabetes in the U.S. was estimated as $98 billion in 1997 comprising $44 billion for direct medical costs such as inpatient and outpatient care plus $54 billion for indirect costs such as lost earnings and productivity, and premature death. Medical innovations that can slow the progression of diabetes therefore have tremendous potential to mitigate the associated clinical and cost repercussions (American Diabetes Association, "Economic consequences of diabetes in the US in 1997," *Diabetes Care* 21:296-309(1998)).

Elevated blood glucose levels are now regarded as causative of diabetic complications based on results of the Diabetes Complications and Control Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) (*N Eng J Med.* 379:977-85 (1993) and *Lancet* 352:837-53 (1998)). The DCCT and the UKPDS have demonstrated that the development of complications of diabetes are related with degree of hyperglycemia and that long-term outcome may be ameliorated by rigorous treatment. For example, prognosis is dramatically improved if capillary blood glucose and glycated hemoglobin levels are maintained less than 150mg/dL and 7.0% respectively.

Glucose condenses with free amino groups on structural and functional proteins to form Schiff bases which, in turn, undergo a series of transformations to yield dark-brown Maillard products. Although the mechanism of glucose toxicity in the tissues of patients with diabetes mellitus is unknown, it has been proposed that diabetes complications are caused by the non-enzymatic cross-linking of proteins. (Cerami A, Ulrich PC, Brownlee M, US Patent 4758583 (1988)). However, although increased protein cross-linking is seen in the tissues of people with long-standing diabetes, the role of Maillard products as a causative factor is not clear (Wolff SP, Jiang ZY, Hunt JV. *Free Rad Biol Med* 10;339-52 (1991)).

Glycation is a post-translational modification of of proteins resulting from condensation of reduced sugars with ε-amino groups of lysine residues on proteins. A

Shiff base intermediate is formed, which undergoes rearrangement to form a relatively stable ketoamine known as an Amadori product. Both the initial formation of a Schiff base moiety and the subsequent rearrangement to form the Amadori product or fructosamine are reversible reactions, which proceed to equilibrium so that the concentration of the Amadori product is proportional to glucose levels in the preceeding period. Amadori-rearrangement is a significant aspect of the Maillard transformation because its product, fructosyl-modified proteins, are the precursors of advanced glycation endproducts (AGEs). Collectively, fructosyl-modified proteins and AGEs, in turn, are termed non-enzymatic glycation products (NEGs) and may be degraded to free proteins in a further reaction, in which reactive oxygen species are generated as reaction products. The reactive oxygen species may cause many of the recognized sequelae of diabetes and impaired glucose tolerance (IGT) if generated in excess. For example, see Nishikawa, T., Edelstein, D., Du, X-L, *et al.*, *Nature* 404: 787-790 (2000).

Several enzymes which interact with NEGs have been described. One group of enzymes said to interact with NEGs to generate reactive oxygen species and can be referred to, *inter alia*, as "fructosyl amine-oxygen oxidoreductases" (or FAOORases; EC 1.5.3). See, for example, Enzyme Nomenclature, Recommendations of the Nomenclature Committee of the International Union of Biochemistry, Academic Press, London pp.19-22 (1979). FAOORase enzymes are copper metalloenzymes that belong to the copper amine oxidase group of enzymes which catalyze the elimination of NEGs from glycated proteins. Some of these enzymes have previously been isolated from bacteria, fungi, and yeast. See, for example, Gerhardinger C., *et al.*, *J Biol Chem* 270(1):218-24 (1995) and Saxena, A.K. *et al.*, *J Biol Chem* 271(51):32803-9 (1996). Other EC1.5.3 FAOORase enzymes have been isolated from mammals. See, for example, WO 00/18392 and WO 00/18891. Products of the FAOORase catalyzed reaction are reportedly free unglycated protein, -dicarbonyl sugar, and the reactive oxygen species superoxide. Other enzymes that utilize synthetic fructosyl moieties as substrates include fructosamine-3-kinase (Delpierre, G., *et al. Diabetes* Oct;49(10):1627-34 (2000), Swergold, B. S., *et al.*, *Diabetes* 50: 2139-2147 (2001)) and FAOORases. A human and a mouse isoform are the only two representatives of this fructosamine-3-kinase family to be fully described, although there is some evidence for the existence of isoforms in a number of other species (Kappler, F., *et al.*, *Diabetes Technology and*

Therapeutics 3(4): 609-616 (2001)). A recent study has proposed that this phosphorylation activity of this enzyme mediates the removal of the sugar moiety from the Amadori product, resulting in the regeneration of the unmodified protein with the concomitant release of the 3-deoxyglucosone (Delpierre, G., *et al.*, *Biochemical Journal* 365(3): 801-808 (2002)).

Some FAOORases have been reported to degrade low molecular mass fructosamines. See, for example, Horiuchi T, *et al.*, *Agric. Biol. Chem.* 53(1):103-110 (1989); Takahashi M., *et al.*, *J. Biol. Chem.* 272: 12505-12507 (1997); and Takahashi M., *et al.*, *J. Biol. Chem.* 272: 3437-3443 (1997). Increased FAOORase activity may cause many of the recognized sequelae of diabetes by degrading NEGs bound to basement membrane proteins and generating reactive oxygen species as reaction products. An alternative explanation for the contribution of FAOORase activity to these processes may reflect increased concentrations of FAOORase substrates in the tissues of at risk individuals. For example, superoxide anions cause an increase in intracellular calcium which modulates the activity of nitric oxide synthase. Nitric oxide is a potent vasodilator and it has been implicated in the vascular dysfunction of early diabetes. See, for example, Ido Y., Kilo C., Williamson J.R. *Nephrol Dial Transplant* 11 Suppl 5:72-5 (1996). Reactive oxygen species also cause a drastic dose-dependent decrease in *de novo* synthesis of heparin sulfate proteoglycans leading to a reduction in anionic sites on the glomerular basement membrane and an increase in basement membrane permeability to cationic plasma proteins such as albumin. See, Kashira N., Watanabe Y., Makin H., Wallner E.I., and Kanwar Y.S., *Proc Natl Acad Sci USA* 89:6309-13 (1992). Increased urinary albumin clearance is a risk indicator in people with diabetes mellitus both for evolving renal disease and for early mortality mainly from coronary heart disease. See, for example, Mattock M.B., Barnes D.J., Viberti G.C., *et al.*, *Diabetes 47:1786-92 (1998).*

Once natural anti-oxidant defenses are exceeded, hydroxyl radicals may be generated from superoxide via a copper catalyzed Haber-Weiss reaction. See, Halliwell B. and Gutteridge J.M.C., "Free radicals in Biology and Medicine" Clarendon Press, Oxford pp. 136-76 (1989). Hydroxyl radicals are extremely reactive species and could cause the permanent site-specific damage to basement membrane proteins and histopathological changes that are typical of diabetic microvascular disease. See, for

example, Robbins S.L., Cotran R.S., Kumar V., "Pathologic basis of disease" 3rd ed. W.B. Saunders, pp. 991-1061. (1984).

Similarly, any prolonged increase in FAOORase activity may result in oxidative stress which could account at least in part for the excess risk of macrovascular disease and the 75% increase in mortality seen in patients with diabetes mellitus compared with non-diabetic individuals. Recent studies have convincing demonstrated that oxidative modification of low density lipoprotein (LDL) is involved in the development of atherosclerosis of coronary and peripheral arterial vessels and elevated oxidized LDL concentrations are found in subjects with diabetes mellitus. See, for example, Witztum J.L., *Br Heart J* 69 (Suppl):S12-S18 (1993) and Picard S., Talussot C., Serusclat A. *et al.*, *Diabetes and Metabolism* 22:25-30 (1996). Oxidative changes to membrane lipids and to membrane protein SH-groups may also cause aberrations in cellular calcium homeostasis and contribute to the increased incidence of cardiac sudden death that is typical of diabetes. See, for example, Yucel D., Aydogdu S., Cehreli S. *et al.*, *Clin Chem* 44:148-54 (1998).

The ability to manipulate, for example, NEGs degradation within an individual is believed to be important in the overall context of the health of the individual and in particular an individual suffering from or predisposed to diabetes mellitus. The discovery and characterization of enzymes that contribute to the degradation of fructosamine and any consequences that result therefrom, such as generation of reactive free radicals, are important for physiological treatments and prophylaxis in disease states including , for example, those which involve NEGs degradation. There exists a need for the isolation and characterization of such enzymes.

SUMMARY OF THE INVENTION

Described herein are novel proteins capable of facilitating modification of glycoproteins and/or other sugar-bearing proteins.

The proteins of the invention participate in the direct or indirect degradation of various molecules, for example, molecules comprising low molecular weight amines and amino acids including fructosamines and other sugar amines, as well as NEGs, AGEs and glycoproteins or other sugar-bearing proteins. While the inventors do not wish to be

bound by any particular theory or mechanism, it is believed that such proteins, termed "Deglycating Enzymes," may modify, for example, NEGs, AGEs and glycoproteins or other sugar-bearing proteins by a mechanism involving activation of one or more modified amino acid sidechains. An example of such a mechanism is the addition of a phosphate group to, for example, the sugar moiety of NEGs, AGEs, and glycoproteins or other sugar-bearing proteins. Such modifications can prime the modified NEGs, AGEs, and glycoproteins or other sugar-bearing proteins for further enzymatically-catalyzed or non-enzymatic degradation or modification.

Alternatively Deglycating Enzymes may effect or participate in this process by the removal of a sugar moiety directly thereby resulting in the release of the unmodified or partially modified protein as a product.

Deglycating Enzyme reactions may also lead to the generation of reactive oxygen species, such as hydrogen peroxide, superoxide, or hydroxyl radical, for example, thereby contributing to oxidative damage which results from, for example, conversion of NEGs or AGEs or glycoproteins or other sugar-bearing proteins into free proteins or proteins with less sugar moieties.

The invention encompasses various polypeptides, examples of which are set forth in sequences comprising, consisting essentially of, or consisting of, SEQ ID NO:1.

The invention further encompasses a polypeptide selected from the group consisting of polypeptides that comprise, consist essentially of, or consist of, an amino acid sequence according to SEQ ID NO:1 with one or more amino acid substitutions, preferably conservative amino acid substitutions.

The polypeptide according to SEQ ID NO:1 has been shown to have enzymatic activity. In one aspect, the present invention provides polypeptides selected from the group consisting of polypeptides that comprise, consist essentially of, or consist of, the following amino acid sequence:

```
MEELLRRELG CSSVRATGHS GGGCISQGRS YDTDQGRVFV KVNPKAEARR 50
MFEGEMASLT AILKTNTVKV PKPIKVLDAP GGGSVLVMEH MDMRHLSSHA 100
AKLGAQLADL HLDNKKLGEM RLKEAGTVGR GGGQEERPFV ARFGFDVVTC 150
CGYLPQVNDW QEDWVVFYAR QRIQPQMDMV EKESGDREAL QLWSALQLKI 200
PDLFRDLEII PALLHGDLWG GNVAEDSSGP VIFDPASFYG HSEYELAIAG 250
```

MFGGFSSSFY SAYHGKIPKA PGFEKRLRLY QLFHYLNHWN HFGSGYRGSS 300
LNIMRNLVK 309(SEQ ID NO:1)

In one aspect, the polypeptide comprises a variant of SEQ ID NO:1 comprising a substitution of isoleucine to valine in the amino acid at position 62 in SEQ ID NO:1. In another aspect, the polypeptide comprises a variant of SEQ ID NO:1 comprising a substitution of arginine to glutamine in the amino acid at position 278 in SEQ ID NO:1. In still another aspect, the polypeptide comprises a variant of SEQ ID NO:1 comprising two amino acid substitutions, an isoleucine to valine substitution at position 62 in the amino acid sequence and an arginine to glutamine substitution at position 278 in the amino acid sequence of SEQ ID NO:1. The polypeptides comprising these amino acid substitutions can be encoded by a polynucleotide comprising one or more nucleotide changes for each amino acid substitution.

In another aspect, the present invention provides a polypeptide which is an analog of SEQ ID NO:1. Analogs of a polypeptide according to SEQ ID NO:1 include, but are not limited to, polypeptides that are homologous to SEQ ID NO:1 and these analogs typically possess a desired property or activity. For example, the present invention provides a polypeptide of SEQ ID NO:1 or an analog thereof which possess one or more of the following properties: (1) apparent molecular weight in the range of 20-75 kDa; (2) ability to modify glycoproteins or other sugar-bearing proteins, directly or indirectly; (3) ability to deglycate or otherwise remove sugars from glycoproteins or other sugar-bearing proteins, directly or indirectly; (4) ability to bind or otherwise interact directly or indirectly with low molecular weight amines and amino acids including fructosamines and other sugar amines, or NEGs or AGEs or glycoproteins or other sugar-bearing proteins; (5) ability to bind and/or effect or participate in the degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins; (6) ability to bind and/or effect or participate in the degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins to produce reactive oxygen species; (7) ability to function as a kinase through, for example, phosphorylation of low molecular weight amines and amino acids including

fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins.

In yet another aspect, the present invention provides a nucleotide sequence encoding a polypeptide of SEQ ID NO:1 or a functional variant thereof. In one aspect, a polypeptide according to SEQ ID NO:1 is encoded by a polynucleotide comprising the following nucleic acid sequence:

```
1     gcggccgcgg cgggaacatg gaggagctgc tgaggcgcga gctgggctgc agctctgtca
61    gggccacggg ccactcgggg ggcgggtgca tcagccaggg ccggagctac gacacggatc
121   aaggacgagt gttcgtgaaa gtgaacccca aggcggaggc cagaagaatg tttgaaggtg
181   agatggcaag tttaactgcc atcctgaaaa caaacacggt gaaagtgccc aagcccatca
241   aggttctgga tgccccaggc ggcgggagcg tgctggtgat ggagcacatg gacatgaggc
301   atctgagcag tcatgctgca aagcttggag cccagctggc cgatttacac cttgataaca
361   agaagcttgg agagatgcgc ctgaaggagg cgggcacagt ggggagagga ggtgggcagg
421   aggaacggcc ctttgtggcc cggtttggat ttgacgtggt gacgtgctgt ggatacctcc
481   cccaggtgaa tgactggcag gaggactggg tcgtgttcta tgcccggcag cgcattcagc
541   cccagatgga catggtggag aaggagtctg gggacaggga ggccctccag ctttggtctg
601   ctctgcagtt aaagatccct gacctgttcc gtgacctgga gatcatccca gccttactcc
661   acggggacct ctggggtgga aacgtagcag aggattcctc tgggccggtg atttttgacc
721   cagcttcttt ctacggccac tcggaatatg agctggcaat agctggcatg tttgggggct
781   ttagcagctc cttttactcc gcctaccacg gcaaaatccc caaggcccca ggattcgaga
841   agcgccttcg gttgtatcag ctctttcact acttgaacca ctggaatcat tttggatcgg
901   ggtacagagg atcctccctg aacatcatga ggaatctggt caagtgagcg ggccttactc
961   tggaaggagg cctcagaggt ttctccacag tcctcttctg ggcaaattct tgtttcttca
1021  catgccggac tagcttaaga ccaatgcagt agcttatttc caagccttgc aaagtatata
1081  atatctaaga ggaaaggttt tgtcatccca gcgttgtcca ctttgtgggg ctttgtaggt
1141  agacggagcc acactacagg cagggtatga gcagagggat gtatggagtg tgggtgactc
1201  tgagcctcac tgctgctgca aggtggggaa actgtaagtg aacccctgtg ggtgcggggg
1261  agggtatccg gtgcgcaggg aggtggccag cgcccccggg cactgctgct cataggtacc
1321  tttccactgc ctcctccctg ctctcctgtg caggaatgtc tctgagctgt tcacgttgat
1381  gcttcttggt tggcaagact tgggtgtaga catgaaacca tcttactaaa agtgtcttaa
1441  aatgaccaat tccagaatca agcgtattcc gttttcttcc tgcatgatcc ctgggccctc
1501  ccgcaggctg agcaagtctg taaactgatt ctgggagaaa ccaagctgct ggccataggg
1561  tgtccttggg tacatccagg agtcttcatt gcttctgtta ttaccccgtc tcctctgcca
1621  ttttctacag cttgctgagt tgtcattcct ttgcaacatt aaaatacatg ctgaactc
```

(SEQ ID NO:2)

In yet another aspect, the present invention provides a polynucleotide that encodes a Deglycating Enzyme of SEQ ID NO:1 or an analog thereof.

In another aspect, the present invention provides a polynucleotide that codes for a bioactive or functional fragment of the Deglycating Enzyme of SEQ ID NO:1 or an analog thereof.

In another aspect, the present invention provides a polynucleotide that encodes a polypeptide which is homologous to the Deglycating Enzyme of SEQ ID NO:1 or an analog thereof wherein the polypeptide binds or otherwise interacts with, directly or indirectly, NEGs or AGEs or glycoproteins or other sugar-bearing proteins and antagonizes the catalyzed degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins.

In another aspect, the present invention provides a polypeptide that is encoded by a polynucleotide that codes for a Deglycating Enzyme of SEQ ID NO:1 or an analog thereof.

In another aspect, the present invention provides a polypeptide that is encoded by a polynucleotide that codes for a bioactive or functional fragment of the Deglycating Enzyme of SEQ ID NO:1 or an analog thereof.

In another aspect, the present invention provides a polypeptide that is capable of modifying, directly or indirectly, a sugar moiety on a polypeptide comprising said sugar moiety. In one aspect, said modification includes the phosphorylation of a sugar moiety on a polypeptide comprising said sugar moiety.

In another aspect, the present invention provides a polypeptide that is homologous to Deglycating Enzyme of SEQ ID NO:1 or an analog thereof wherein the polypeptide binds to or otherwise interacts with, directly or indirectly, NEGs or AGEs or glycoproteins or other sugar-bearing proteins and antagonizes the degradation process of the NEGs or AGEs or glycoproteins or other sugar-bearing proteins.

In another aspect, the present invention provides a vector that comprises a polynucleotide that encodes any of the polypeptides described or referenced herein, including the Deglycating Enzyme of SEQ ID NO:1 or an analog thereof and that is capable of, for example, expressing said polypeptides including the Deglycating Enzyme of SEQ ID NO:1 or said analog(s).

In another aspect, the present invention provides a vector which comprises a polynucleotide that encodes a bioactive or functional fragment of the Deglycating Enzyme of SEQ ID NO:1 or an analog thereof.

In another aspect, the present invention provides a vector which comprises a polynucleotide that encodes a polypeptide which is homologous to Deglycating Enzyme of SEQ ID NO:1 or an analog thereof wherein the polypeptide binds to or otherwise interacts with, directly or indirectly, NEGs or AGEs or glycoproteins or other sugar-bearing proteins and antagonizes the degradation process of the NEGs or AGEs or glycoproteins or other sugar-bearing proteins.

In yet another aspect, the present invention provides for a host cell comprising a vector that comprises any of the vectors described or referenced herein, including, for example, vectors comprising one or more polynucleotides that encode a Deglycating Enzyme of SEQ ID NO:1, a bioactive or functional fragment of the Deglycating Enzyme of SEQ ID NO:1, an analog or variant thereof, or a bioactive or functional fragment or other fragment thereof.

In yet another aspect, the present invention provides for a host cell comprising a vector that comprises a polynucleotide encoding a polypeptide that is homologous to a Deglycating Enzyme of SEQ ID NO:1 or an analog thereof wherein the polypeptide binds to or otherwise interacts with, directly or indirectly, NEGs or AGEs or glycoproteins or other sugar-bearing proteins and antagonizes the degradation process of the NEGs and/or AGEs or glycoproteins or other sugar-bearing proteins

In yet another aspect, the present invention provides for salts comprising any of the polypeptides described or referenced herein, including, for example, the Deglycating Enzyme of SEQ ID NO:1 or analogs or variants thereof.

In yet another aspect, the present invention provides methods for screening for and/or identifying agents that modulate one or more activities of a Deglycating Enzyme comprising, for example: determining the amount of low molecular weight sugar moieties or reactive oxygen species or NEGs and/or AGEs or glycoproteins or other sugar-bearing proteins in a biological sample from an individual; selecting an agent or agents for testing; administering a pre-determined amount of the agent or agents to the individual; comparing the amount of low molecular weight sugar moieties or reactive oxygen species or NEGs and/or AGEs or glycoproteins or other sugar-bearing proteins

in pre-administration samples and post-administration samples to determine the extent to which the agent modulates one or more Deglycation Enzyme activities, for example, kinase activity.

In yet another aspect, the present invention provides methods for screening for and/or identifying agents that modulate the activity of Deglycating Enzyme in a cell, cell line, transfected cell line, or an isolated tissue sample, comprising, for example: determining the amount of low molecular weight sugar moieties, reactive oxygen species, or NEGs and/or AGEs or glycoproteins or other sugar-bearing proteins in a cell, cell line, transfected cell line, or an isolated tissue sample, for example; selecting one or more agents for testing; administering a pre-determined amount of the agent or agents; comparing the amount of low molecular weight sugar moieties, reactive oxygen species, or NEGs and/or AGEs or glycoproteins or other sugar-bearing proteins in the pre-administration cell, cell line, transfected cell line, or an isolated tissue sample with the amount of low molecular weight sugar moieties, reactive oxygen species, or NEGs and/or AGEs or glycoproteins or other sugar-bearing proteins in the post-administration cell, cell line, transfected cell line, or an isolated tissue sample to determine the extent to which the agent modulates one or more Deglycation Enzyme activities, for example, kinase activity.

In yet another aspect, the present invention provides methods for screening for and/or identifying agents that antagonize the activity of Deglycating Enzyme comprising, for example: selecting an agent for testing; admixing a pre-determined amount of the agent with Deglycating Enzyme; determining the amount of free NEGs or AGEs or glycated protein and/or reactive oxygen species; selecting the agent that results in lowered reactive oxygen species or reduced NEGs and/or AGEs degradation or otherwise antagonizes one or more Deglycation Enzyme activities, for example, kinase activity.

In yet another aspect, the present invention provides a method of screening for drugs comprising monitoring Deglycating Enzyme activity to identify an agent that antagonizes Deglycating Enzyme activity.

In yet another aspect, the present invention provides a method of determining the modulatory activity (*e.g.*, inhibitory or activating) of a candidate agent or agents comprising, for example, determining the rate and/or amount of NEGs and/or AGEs

degradation or amount of reactive oxygen species generated prior to exposure to the candidate agent and comparing this rate to the rate of NEGs and/or AGEs degradation or amount of reactive oxygen species generated after exposure to the candidate agent or agents.

In yet another aspect, the present invention provides for a pharmaceutical composition that comprises one or more of the bioactive polypeptides described or referenced herein, including, for example, a Deglycating Enzyme of SEQ ID NO:1 or an analog thereof or functional variant or fragment thereof, or salts which comprise the Deglycating Enzyme of SEQ ID NO:1, analog, variant or fragment.

In another aspect, the present invention provides a method of providing therapy to an individual who has complications associated with diabetes comprising administering to the individual an effective amount of one or more of the bioactive polypeptides described or referenced herein, including, for example, a Deglycating Enzyme of SEQ ID NO:1 or an analog thereof or functional variant or fragment thereof, or salts which comprise the Deglycating Enzyme of SEQ ID NO:1, analog, variant or fragment.

In still a further aspect, the present invention provides a method of providing therapy to an individual who is in need of such therapy comprising administering to the individual an effective amount of one or more of the bioactive polypeptides described or referenced herein, including, for example, a Deglycating Enzyme of SEQ ID NO:1 or an analog thereof or functional variant or fragment thereof, or salts which comprise the Deglycating Enzyme of SEQ ID NO:1, analog, variant or fragment.

In still a further aspect, the present invention provides a method of treating an individual with a condition which results in or involves undesired amount of NEGs or AGEs and/or glycoproteins or other sugar-bearing proteins comprising administering to the individual an effective amount of one or more of the bioactive polypeptides described or referenced herein, including, for example, a Deglycating Enzyme of SEQ ID NO:1 or an analog thereof or functional variant or fragment thereof, or salts which comprise the Deglycating Enzyme of SEQ ID NO:1, analog, variant or fragment.

In another aspect, the present invention provides methods for delaying development of complications associated with diabetes in an individual comprising administering to the individual an effective amount of one or more of the bioactive

polypeptides described or referenced herein, including, for example, a Deglycating Enzyme of SEQ ID NO:1 or an analog thereof or functional variant or fragment thereof, or salts which comprise the Deglycating Enzyme of SEQ ID NO:1, analog, variant or fragment.

In yet another aspect, the present invention provides methods for delaying development of a disease state that is associated with excessive amount of of of NEGs or AGEs and/or glycoproteins or other sugar-bearing proteins comprising comprising administering to an individual an effective amount of one or more of the bioactive polypeptides described or referenced herein, including, for example, a Deglycating Enzyme of SEQ ID NO:1 or an analog thereof or functional variant or fragment thereof, or salts which comprise the Deglycating Enzyme of SEQ ID NO:1, analog, variant or fragment.

In still a further aspect, the present invention provides a method of modulating NEGs or AGEs or glycoproteins or other sugar-bearing proteins or the glycation of proteins comprising comprising administering to an individual an effective amount of one or more of the bioactive polypeptides described or referenced herein, including, for example, a Deglycating Enzyme of SEQ ID NO:1 or an analog thereof or functional variant or fragment thereof, or salts which comprise the Deglycating Enzyme of SEQ ID NO:1, analog, variant or fragment.

The invention also provides a method of treating a subject for hyperglycemia comprising administering to said subject a therapeutically effective amount of a polypeptide selected from the group consisting of a polypeptide comprising the amino acid sequence of SEQ. ID NO: 1, a variant of the amino acid sequence of SEQ. ID NO: 1, an analog of the amino acid sequence of SEQ. ID NO: 1, a homolog of the amino acid sequence of SEQ. ID NO: 1, a bioactive or functional fragment of the amino acid sequence of SEQ. ID NO: 1, and other bioactive polypeptides described or referenced or provided herein.

The invention also provides a method of treating a subject for diabetes or one or more complications associated with diabetes comprising administering to said subject a therapeutically effective amount of a polypeptide selected from the group consisting of a polypeptide comprising the amino acid sequence of SEQ. ID NO: 1, a variant of the amino acid sequence of SEQ. ID NO: 1, an analog of the amino acid sequence of SEQ.

ID NO: 1, a homolog of the amino acid sequence of SEQ. ID NO: 1, a bioactive or functional fragment of the amino acid sequence of SEQ. ID NO: 1, and other bioactive polypeptides described or referenced or provided herein.

The invention also provides a method of treating a subject with an elevated glycated hemoglobin level comprising administering to said subject a therapeutically effective amount of a polypeptide selected from the group consisting of a polypeptide comprising the amino acid sequence of SEQ. ID NO: 1, a variant of the amino acid sequence of SEQ. ID NO: 1, an analog of the amino acid sequence of SEQ. ID NO: 1, a homolog of the amino acid sequence of SEQ. ID NO: 1, a bioactive or functional fragment of the amino acid sequence of SEQ. ID NO: 1, and other bioactive polypeptides described or referenced or provided herein. In another aspect, the subject has a glycated hemoglobin level greater than about 7.0%.

The invention also provides a method of treating a subject having or suspected of having an undesired amount of glycated proteins comprising administering to said subject a therapeutically effective amount of a polypeptide selected from the group consisting of a polypeptide comprising the amino acid sequence of SEQ. ID NO: 1, a variant of the amino acid sequence of SEQ. ID NO: 1, an analog of the amino acid sequence of SEQ. ID NO: 1, a homolog of the amino acid sequence of SEQ. ID NO: 1, a bioactive or functional fragment of the amino acid sequence of SEQ. ID NO: 1, and other bioactive polypeptides described or referenced or provided herein. In another aspect, the glycated proteins refered to in this method comprise advanced glycation endproducts. In yet another aspect, the glycated proteins refered to in this method comprise non-enzymatic glycation products.

In still another aspect, the present invention provides for a pharmaceutical composition that comprises an agent or agents that antagonize the activity of Deglycating Enzyme of SEQ ID NO:1 or an analog thereof, or salts which comprise the Deglycating Enzyme of SEQ ID NO:1. Such agents include antibodies and antisense sequences. In another aspect, the present invention provides a method of providing therapy to an individual who has complications associated with diabetes comprising administering to the individual an effective amount of an agent that antagonizes a Deglycating Enzyme or an analog thereof. In still a further aspect, the present invention provides a method of providing therapy to an individual who is in need of such therapy

comprising administering to the individual an effective amount of an agent that antagonizes a Deglycating Enzyme or an analog thereof. In still a further aspect, the present invention provides a method of treating an individual with a condition which results in or involves undesired amount of degradation of NEGs or AGEs and/or glycoproteins or other sugar-bearing proteins comprising administering to the individual an effective amount of an agent that antagonizes a Deglycating Enzyme or an analog thereof. In another aspect, the present invention provides methods for delaying development of complications associated with diabetes in an individual comprising administering to the individual an effective amount of an agent that antagonizes a Deglycating Enzyme or an analog thereof. In yet another aspect, the present invention provides methods for delaying development of a disease state that is associated with excessive amount of of degradation of NEGs or AGEs and/or glycoproteins or other sugar-bearing proteins comprising comprising administering to an individual an effective amount of an agent that antagonizes a Deglycating Enzyme or an analog thereof. In still a further aspect, the present invention provides a method of modulating degradation of NEGs or AGEs and/or glycoproteins or other sugar-bearing proteins comprising comprising administering to an individual an effective amount of an agent that antagonizes a Deglycating Enzyme or an analog thereof. Agents useful in the above methods include, for example, antibodies and antisense sequences.

In yet another aspect, the present invention provides antibodies that bind a Deglycating Enzyme or its analogs. In still another aspect, the present invention provides antibodies that specifically bind a Deglycating Enzyme or its analogs, said binding specificity being to a desired degree or to a degree that, under conditions of intended use, the antibody does not otherwise undesireably interact with non-target antigens. Antibodies generated against the polypeptides of the invention can be obtained by administering the polypeptides or epitope-bearing fragments, analogs or cells to an animal, preferably a nonhuman, using routine protocols. For preparation of monoclonal antibodies, any technique known in the art that provides antibodies produced by continuous cell line cultures can be used. Examples include various techniques, such as those in Kohler, G. and Milstein, C., *Nature* 256: 495-497 (1975); Kozbor *et al.*, *Immunology Today* 4: 72 (1983); Cole *et al.*, pg. 77-96 in MONOCLONAL ANTIBODIES AND CANCER THERAPY, Alan R. Liss, Inc. (1985). Thus, in another

aspect, the present invention provides methods for generating antibodies reactive against Deglycating Enzyme comprising, for example: immunizing a host mammal with a Deglycating Enzyme or a Deglycating Enzyme analog or fragment, for example, which may be conjugated or unconjugated; obtaining lymphocytes from said mammal; fusing lymphocytes obtained from said mammal with a myeloma or other appropriate or desired cell line to produce a hybridoma; culturing said hybridoma to produce monoclonal antibodies; screening the antibodies to select those antibodies that bind to a desired Deglycating Enzyme or Deglycating Enzyme analog or fragment, for example. Techniques for the production of single chain antibodies (U.S. Pat. No. 4,946,778) can be adapted to produce single chain antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms such as other mammals, may be used to express humanized antibodies. Alternatively, for example, phage display technology may be utilized to select antibody genes with binding activities towards the polypeptide either from repertoires of PCR amplified v-genes of lymphocytes from humans screened for possessing anti-Histidine Kinase or from naive libraries (McCafferty, J. *et al.*, (1990), *Nature* 348, 552-554; Marks, J. *et al.*, (1992) *Biotechnology* 10, 779-783). The affinity of these antibodies can also be improved by chain shuffling (Clackson, T. *et al.*, (1991) *Nature* 352, 624-628).

In yet another aspect, the present invention provides methods for identifying for proteins (*e.g.*, receptors, agonists, antagonists, transcription factors, *etc.*) that interact with a Deglycating Enzyme comprising admixing a Deglycating Enzyme with a biological sample and detecting the interaction of the Deglycating Enzyme with one or more components of the biological sample.

In yet another aspect, the present invention provides methods for identifying for nucleic acids that interact with a Deglycating Enzyme comprising admixing Deglycating Enzyme with a biological sample and detecting the interaction of Deglycating Enzyme with one or more components of the biological sample.

In yet another aspect, the present invention provides methods for identifying transcriptional and/or translational variants of Deglycating Enzyme comprising using a portion of Deglycating Enzyme sequence as a probe for screening animal genomes.

The invention also provides for Deglycating Enzyme antisense sequences.

The invention also provides for assays for a Deglycating Enzyme or variant thereof, including fragments, analogs, and such. The antibodies of the present invention may be employed in any known assay method or formats, including for example, immunoassays and formats such as the RIA or ELISA, and may use detectably labeled antibodies. Other types of assays include but are not limited to, other competitive-binding assays, direct and indirect sandwich assays, and immunoprecipitation assays, Western Blot analysis, fluorescence immunoassays, fluorescence polarization immunoassay, and other assay techniques that are well-known to those of skill in the art. Zola, Monoclonal Antibodies: A Manual of Techniques, pp.147-158 (CRC Press, Inc., 1967).

In another aspect, the invention features kits for performing the above-described assays. The kit can include sample collection means known in the art and a means for determining at least one Deglycating Enzyme or related polypeptide, such means including any of various known assays or immunoassays. The kit may also comprise control samples or standards. The kit will also typically comprise instructions on use.

Information obtained using the assays and kits described herein (alone or in conjunction with information) is useful for research and for evaluating whether a subject is likely to respond to one or more of the therapies described or referenced herein. In addition, the information alone or in conjunction with information allows customization of therapy to the individual. For example, this information can enable a doctor to: (1) more effectively prescribe a drug that will address the molecular basis of the disease or condition for which treatment is indicated; and (2) better determine the appropriate dosage of a particular drug for a particular patient.

For therapeutic applications, the antibodies of the invention are administered to a mammal, preferably a human, in a pharmaceutically acceptable dosage form. They are administered intravenously as a bolus or by continuous infusion over a period of time, by intramuscular, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. When the antibody possesses the suitable activity it is also suitably administered by intratumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects.

The antibodies of the invention also are useful as affinity purification agents. In one example of such a process, antibodies against a Deglycating Enzyme are

immobilized on a suitable support, such as Sephadex resin or filter paper, using methods well known in the art. The immobilized antibodies are then is contacted with a sample containing the protein to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the desired, which is bound to the immobilized antibodies. Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0, that will release the desired protein from the antibody.

Dosage forms encompass pharmaceutically acceptable carriers that are inherently nontoxic and nontherapeutic. Examples of such carriers include ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffers such as phosphate or glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts, or electrolytes such as protamine sulfate, sodium chloride, metal salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulosic polymers, and polyethylene glycol. Carriers for topical or gel-based forms of antibody include polysaccharides such as sodium carboxymethylcellulose or methylcellulose, polyvinylpyrrolidone, polyacrylates, polyoxyethylene-polyoxypropylene-blockpolymers, polyethylene glycol, and wood wax alcohols. Conventional depot forms include, for example, microcapsules, nano-capsules, liposomes, plasters, sublingual tablets, and polymer matrices such as polylactide:polyglycolide copolymers. When present in an aqueous dosage form, rather than being lyophilized, the antibody typically will be formulated at a concentration of about 0.1 mg/ml to 100 mg/ml, although wide variation outside of these ranges is permitted.

According to another embodiment of the invention, the effectiveness of the pharmaceutical compositions described hering in preventing or treating disease may be improved by administering the composition in combination with another agent that is useful or effective for the same clinical objective, such as one or more conventional therapeutic agents known for the intended therapeutic indication, *e.g.* prevention or treatment of conditions associated with glycation of proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts amplification of a PCR cDNA fragment which encodes an enzyme with Deglycating Enzyme activity.

Figure 2 depicts purification of a 556 bp (base pair) PstI restriction digestion fragment of a Deglycating Enzyme.

Figure 3 depicts Northern blot of total RNA isolated from human tissue probed with purified 556 bp radiolabelled probe showing expression in several tissues of a specific mRNA corresponding to a Deglycating Enzyme.

Figure 4 depicts multiple sequence alignment of a Deglycating Enzyme with predicted proteins from known genomes. Alignments were performed using ClustalW 1.8. Proteins from the top are as follows: /usr/tmp/aaaa05482 = human Deglycating Enzyme (NCBI accession No. AK922233); /usr/tmp/aaaa05592 = human fructosamine-3-kinase (NCBI Accession No. AJ404615); /usr/tmp/aaaa05254 = putative protein based on *C. elegans* genome (EMBL data library Accession No. T31496); /usr/tmp/aaaa05739 = *Pasteurella multocida* genome (NCBI Accession No. AE006094).

Figure 5 depicts multiple alignment of protein sequences displaying significant homology with the characterised Deglycating Enzyme. The alignment was generated using the ClustalX program. NFNK refers to the sequence of Deglycating Enzyme. AFNK, CFNK, and K12FNK refer to ORFs identified in Arabidopsis thaliana, Ceanorhabditis elegans, and Escherishia coli K12 respectively. Regions of identity are outlined in dark grey. Regions of additional homology are highlighted in light grey. The boxed region outlines the conserved HGDLWGGN (SEQ ID NO: 18) motif. Arrows mark conserved residues that have been hypothesised to correspond to conserved active site residues identified in the aminoglycoside family of kinases.

Figure 6 depicts a typical trace obtained during purification of a Deglycating Enzyme (F3KLPNHis) on Poros HQ-20 media, wherein the red trace is the A_{280}. Fractions collected are marked in blue, and were numbered sequentially from 1-20. F3KLPNHis elutes as a single peak early in the salt gradient, corresponding to fractions 4-8.

Figure 7 depicts SDS-PAGE of fractions taken during purification of a Deglycating Enzyme (F3KLPNHis) from SF-9 Cells. The recombinant enzyme was purified using Cobalt affinity purification followed by Q-sepharose chromatography.

Lane 1: Molecular weight standards; lane 2: cleared cell lysate; lane 3: eluted fraction from cobalt column; lane 4: major peak from Q-sepharose column.

Figure 8 depicts a typical mass spectum from the tryptic digest of a Deglycating Enzyme (F3KLPNHis).

Figure 9 depicts a composite of western blots performed to test the reactivity against recombinant Deglycating Enzyme isoforms. Lanes 1-4 were loaded with 2μg of purified F3KLPNHis. Lane 5 was loaded with a lysate of untransfected Cos-7 cells. Lane 6 was loaded with a lysate of Cos-7 cells expressing F3KLPCFLAG. Dilutions of serum used were: 1:10 for lane 1; 1:100 for lane 2; 1:1000 for lanes 3,5, and 6; and 1:10000 for lane 4.

Figure 10 depicts measurement of the kinase activity of a Deglycating Enzyme, wherein "GL" are reaction mixtures containing glycated lysozyme as substrate (n=6), "NGL" are reaction mixtures containing non-glycated lysozyme as substrate (n=3), "-L" represents the reaction mixture lacking lysozyme (n=2), and "No DE" is a reaction lacking Deglycating Enzyme (n=6). Values represent means and standard errors from measurements taken from two separate experiments. Statistical significance was determined by one way ANOVA followed by post-hoc analysis by Dunnett's test. ***= $P < 0.001$ vs GL.

DETAILED DESCRIPTION OF THE INVENTION

The invention is related to and describes the molecular identification and cloning of novel proteins. The invention includes proteins that have deglycating activity. It also includes proteins that function as kinases to phosphorylate other molecules. Proteins of the invention may be refered to as enzymes, although the inventors do not intend to be bound by the term "enzyme." The proteins are believed to have deglycating activities, for example enzymatic deglycating activities, or to participate in such activities, in a process catalyzed entirely though their own activity or in combination with other enzymes or molecules that lead to or participate in deglycation of proteins, including a reduction in the amount of sugar residues attached to proteins. These proteins may, for example, bind to and cleave or otherwise interact directly or indirectly with low molecular weight amines and amino acids including fructosamines, NEGs, AGEs, and/or other sugar amines or glycoproteins or other sugar-bearing proteins.

Experiments described herein have characterized a class of protein enzymes. The proteins may be in a class such as that of FAOORases (EC1.5.3 group). The Deglycating Enzymes described herein are typically about 20-75 kDa in size and are believed to participate in the phosphorylation of sugars on proteins. They may also react with low molecular weight amines and amino acids including fructosamines, NEGs, and other sugar amines, for example, in a manner that may generate undesired reactive oxygen species.

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook *et al.*, 1989) Cold Spring Harbor Press; *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Animal Cell Culture* (R.I. Freshney), ed., 1987); *Methods in Enzymology* (Academic Press, Inc.); *Handbook of Experimental Immunology* (D.M. Weir & C.C. Blackwell, eds.); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller & M.P. Calos, eds., 1987); *Current Protocols in Molecular Biology* (F.M. Ausubel *et al.*, eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis *et al.*, eds., 1994); *Current Protocols in Immunology* (J.E. Coligan *et al.*, eds., 1991) and *Short Protocols in Molecular Biology* (Wiley and Sons, 1999).

Definitions

Before describing the invention in general and in terms of specific embodiments, certain terms used in the context of the describing the invention are set forth. Unless indicated otherwise, the following terms have the following meanings when used herein and in the appended claims. Those terms that are not defined below or elsewhere in the specification shall have their art-recognized meaning.

As used herein, "abnormal Deglycating Enzyme activity" refers to a deviation in Deglycating Enzyme activity from the normal or average. Methods of measuring Deglycating Enzyme activity are described herein. Deviation from normal or average can be in reference to measurements taken over a period of time for one individual. Alternatively, deviation from normal or average can refer to measurements taken at the same time or different time points for a group of individuals. The individuals may be

grouped by various factors, including but not limited to, genetic makeup, treatment protocols administered, presence or absence of disease state (*e.g.*, diabetes), history of disease state (*e.g.*, diabetes), sex, weight, and other physical factors.

As used herein, the term "agent" means a biological or chemical compound such as a simple or complex organic or inorganic molecule, a peptide, a protein, an oligonucleotide, an antibody, an antibody derivative, or antibody fragment. A vast array of compounds can be synthesized, for example oligomers, such as oligopeptides and oligonucleotides, and synthetic organic compounds based on various core structures, and these are also included in the term "agent". Also included in the term "agent" are antibodies which are generated in animals or synthesized recombinantly or by phage display. Further, the term "agent" includes polypeptides or peptides which are homologous to a Deglycating Enzyme and can bind to or otherwise interact directly or indirectly with low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs, or AGEs and/or glycoproteins or other sugar-bearing proteins, for example, in a manner that facilitates or antagonizes their degradation or deglycation. In addition, various natural sources can provide agents for screening, such as plant or animal extracts, and the like. Agents can be tested and/or used singly or in combination with one another.

As used herein, the term "agent which antagonizes the activity of Deglycating Enzyme" refers to any agent which inhibits, reduces, provokes the inhibition or reduction, or interferes with an activity of a Deglycating Enzyme. Generally, administration of an effective amount of "agent which antagonizes the activity of Deglycating Enzyme" results in a greater reduction or inhibition of degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs, or AGEs and/or glycoproteins or other sugar-bearing proteins, for example, than that which results when such an agent is not administered.

The term "agonist" refers to a molecule that has one or more functions of a Deglycating Enzyme or that modulates *in vivo* levels or activites of a Deglycating Enzyme. It may increase or decrease absolute Deglycating Enzyme activity or, alternatively, influence positively or negatively some other parameter of Deglycating Enzyme activity, for example, pH, temperature, or co-factor dependence. A "negative agonist" is a compound that decreases the activity of a protein, while a "positive agonist"

is a compound that increases the activity of a protein. An "antagonist" is a compound that competes with another compound for interactions with a protein functional site. Agonists and antagonists include antibodies, small molecules, proteins, lipids, carbohydrates, and other molecules.

The terms "allele" or "allelic sequence," as used herein, refer to a naturally-occurring alternative form of a gene encoding a Deglycating Enzyme polypeptide (*i.e.*, a polynucleotide encoding an Deglycating Enzyme polypeptide). Alleles often result from mutations (*i.e.*, changes in the nucleic acid sequence), and sometimes produce altered and/or differently regulated mRNAs or polypeptides whose structure and/or function may or may not be altered. Common mutational changes that give rise to alleles are generally ascribed to natural deletions, additions, or substitutions of nucleotides that may or may not affect the encoded amino acids. Each of these types of changes may occur alone, in combination with the others, or one or more times within a given gene, chromosome or other cellular polynucleotide. Any given gene may have no, one or many allelic forms. As used herein, the term "allele" refers to either or both a gene or an mRNA transcribed from the gene.

An "amino acid" is a molecule having the structure wherein a central carbon atom (the "alpha (α)-carbon atom") is linked to a hydrogen atom, a carboxylic acid group (the carbon atom of which is referred to as a "carboxyl carbon atom"), an amino group (the nitrogen atom of which is referred to as an "amino nitrogen atom"), and a side chain group, R. In the process of being incorporated into a protein, an amino acid loses one or more atoms of its amino and carboxylic groups in a dehydration reaction that links one amino acid to another. As a result, when incorporated into a protein, an amino acid is often referred to as an "amino acid residue." An amino acid may be derivatized or modified before or after incorporation into a protein (for example, by glycosylation, by formation of cystine through the oxidation of the thiol side chains of two non-contiguous cysteine amino acid residues, resulting in a disulfide covalent bond that frequently plays an important role in stabilizing the folded conformation of a protein, *etc.*). An amino acid may be one that occurs in nature in proteins, or it may be non-naturally occurring (*i.e.*, is produced by synthetic methods such as solid state and other automated synthesis methods). Examples of non-naturally occurring amino acids include α-amino isobutyric acid, 4-amino butyric acid, L-amino butyric acid, 6-amino

hexanoic acid, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleusine, norvaline, hydroxproline, sarcosine, citralline, cysteic acid, t-butylglyine, t-butylalanine, phenylylycine, cyclohexylalanine, β-alanine, fluoro-amino acids, including beta and gamma amino acids, designer amino acids (for example, β-methyl amino acids, α-methyl amino acids, Nα-methyl amino acids), and amino acid analogs in general. Amino acid analogs refer to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an alpha-carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, for example, but have modified R groups (for example, norleucine) or modified peptide backbones, while retaining the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that generally function in a manner similar to a naturally occurring amino acid.

In addition to its substitutent groups, two different enantiomeric forms of each amino acid exist, designated D and L. In mammals, only L-amino acids are incorporated into naturally occurring proteins, although the invention contemplates proteins incorporating one or more D- and L- amino acids, as well as proteins comprised of just D- or just L- amino acid residues.

Herein, the following abbreviations may be used for the following amino acids (and residues thereof): alanine (Ala, A); arginine (Arg, R); asparagine (Asn, N); aspartic acid (Asp, D); cyteine (Cys, C); glycine (Gly, G); glutamic acid (Glu, E); glutamine (Gln, Q); histidine (His, H); isoleucine (Ile, I); leucine (Leu, L); lysine (Lys, K); methionine (Met, M); phenylalanine (Phe, F); proline (Pro, P); serine (Ser, S); threonine (Thr, T); tryptophan (Trp, W); tyrosine (Tyr, Y); and valine (Val, V).

The term "amino acid sequence" refers to an oligopeptide, peptide, polypeptide, or protein sequence, a fragment of any of these, and to naturally occurring or synthetic molecules, as well as to electronic or other representations of foregoing suitable for use in conjunction with a computer, for example.

As will be appreciated, embodiments of the invention may be implemented *in silico*. In such embodiments, actual physically existing amino acids, peptide fragments, *etc.* are not employed; instead, electronic or other machine manipulable data forms

representing these molecules are used. It is understood that in such embodiments, the foregoing nomenclature, while preferable, need not be used. Instead, any suitable nomenclature for such data forms may be employed.

The term "analog" as used herein generally refers to compounds which are generally structurally similar to the compound of which they are an analog, or "parent" compound. Generally analogs will retain certain characteristics of the parent compound, *e.g.*, one more more biological or pharmacological activities. An analog may lack other, less desirable characteristics, *e.g.*, antigenicity, proteolytic instability, toxicity, and the like. As applied to polypeptides, the term "analog" generally refers to polypeptides which are comprised of at least a segment of amino acids that have substantial identity to at least a portion of a Deglycating Enzyme polypeptide. Analogs typically are at least 50 amino acids long, at least 100 amino acids long or longer, at least 150 amino acids long or longer, at least least 200 amino acids long or longer, and more typically at least 250 amino acids long or longer, or 300 amino acids or longer. Some analogs may lack substantial biological activity but may still be employed for various uses, such as for raising antibodies to predetermined epitopes, as an immunological reagent to detect and/or purify reactive antibodies by affinity chromatography, or as a competitive or noncompetitive agonist, antagonist, or partial agonist of a Deglycating Enzyme function. Analogs include "functional analogs."

An "antibody" (interchangeably used in plural form) is an immunoglobulin molecule or portion thereof capable of specific binding to a target, such as a carbohydrate, polynucleotide or polypeptide, through at least one antigen recognition site, located in the variable region of the immunoglobulin molecule. As used herein, the term encompasses not only intact antibodies, but also fragments thereof (such as Fab, Fab', $F(ab')_2$, Fv), single chain (ScFv), mutants thereof, fusion proteins comprising an antibody portion, humanized antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site of the desired or required specificity. Antibodies include polyclonal antibodies and monoclonal antibodies.

The term "antisense sequences" refers to polynucleotides having a sequence complementary to a RNA sequence. These terms include nucleic acid sequences that bind to mRNA or portions thereof to block transcription of mRNA by ribosomes.

Antisense methods are generally well known in the art (see, for example, PCT publication WO 94/12633, and *Nielsen et al.*, 1991, *Science* 254:1497; OLIGONUCLEOTIDES AND ANALOGS, A PRACTICAL APPROACH, edited by F. Eckstein, IRL Press at Oxford University Press (1991); ANTISENSE RESEARCH AND APPLICATIONS (1993, CRC Press)).

As used herein, "bioactive" refers to possessing a biologically active function, for example, a deglycation activity or a kinase activity. In general, the terms "biologically active", "bioactive", and "functional variant" refer to a protein having one or more functions, for example, a structural, regulatory, or biochemical function, of a naturally occurring molecule. With regard to a Deglycating Enzyme, the terms "biologically active" or "bioactive" refer to a full length protein or fragment thereof derived from any source, or a variant, a derivative or an analog of a full length protein or fragment, that possesses one or more Deglycating Enzyme characteristics including measurable Deglycating Enzyme activity, for example, deglycation or kinase activity. Such full length protein, fragments, variants, derivatives and/or analogs ay also show immunological cross reactivity with an antibody (polyclonal or monoclonal) that is raised against, and reacts with, a Deglycating Enzyme having the amino acid sequence of a naturally occurring Deglycating Enzyme.

A protein's "biochemical activity" refers to a chemical interaction or reaction mediated by or involving the protein. Herein, "biochemical activity" is refers to one or more Deglycating Enzyme activities in general, or a specific type of Deglycating Enzyme activity.

A "biological sample" encompasses a variety of sample types obtained from an individual and can be used in a diagnostic or monitoring assay. The definition encompasses blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom, and the progeny thereof. The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain components, such as proteins or polynucleotides. The term "biological sample" encompasses a clinical sample, and also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples. It is understood that a biological sample can contain a native Deglycating Enzyme.

A "cell" means any living cell suitable for the desired application. Cells include eukaryotic and prokaryotic cells. Preferred eukaryotic cells include vertebrate cells such as mammalian cells (for example, human, murine, ovine, porcine, equine, canine, and feline cells), avian cells, fish cells, and invertebrate cells such as insect cells and yeast cells. Preferred prokaryotic cells are bacterial cells.

A "cell-based assay" is one that employs cells that express a protein of interest. The cell may express the protein endogenously or as a result of recombinant techniques, including the introduction of a suitable expression vector or by introduction (for example, by homologous recombination) of a suitable regulatory element capable of directing the expression of the desired protein. Expression may be constitutive or inducible, as well as transient or stable.

The term "complementary" generally refers to the natural binding of polynucleotides under permissive salt and temperature conditions by base pairing. For example, the sequence "A-G-T" binds to the complementary sequence "T-C-A". Complementarity between two single-stranded molecules may be "partial", such that only some of the nucleic acids bind, or it may be "complete", such that total complementarity exists between the single stranded molecules. The degree of complementarity between nucleic acid molecules has significant effects on the efficiency and strength of the hybridization between them.

The term "composition" as used herein is intended to encompass a product comprising one or more specified ingredients in specified or other amounts, as well as any product which results, directly or indirectly, from combination of the specified ingredients in such specified or other amounts.

A "compound" is a molecule, and includes small molecules, proteins, carbohydrates, and lipids, for example.

A "compound known to interact" with a protein means a compound that has previously been identified as interacting with a protein or other target.

The term "conservative substitution," when describing a polypeptide, refers to a change in the amino acid composition of the polypeptide that does not substantially alter the activity of the polypeptide, *i.e.*, substitution of amino acids with other amino acids having similar properties. Conservative substitution tables providing functionally similar amino acids are well known in the art. Substitutions of particular amino acid

residues based on common side chain properties is also anticipated within the scope of this invention. Naturally-occurring amino acids are divided into groups based on common side chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophobic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, agr;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Conservative substitutions involve exchanging a member within one group for another member within the same group, whereas non-conservative substitutions will entail exchanging a member of one of these classes for another. Variants obtained by non-conservative substitutions may result in significant changes in the biological properties/function of the obtained variant. Amino acid positions that are conserved among various species are generally substituted in a relatively conservative manner if the goal is to retain biological function. See also, Creighton, 1984, *Proteins,* W.H. Freeman and Company.

In addition to the above-defined conservative substitutions, other modifications of amino acid residues can also result in "conservatively modified variants." For example, one may regard all charged amino acids as substitutions for each other whether they are positive or negative. In addition, conservatively modified variants can also result from individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids, for example, often less than 5%, in an encoded sequence. Further, a conservatively modified variant can be made from a recombinant polypeptide by substituting a codon for an amino acid employed by the native or wild-type gene with a different codon for the same amino acid.

The terms "control elements" or "regulatory sequences" include enhancers, promoters, transcription terminators, origins of replication, chromosomal integration sequences, 5' and 3' untranslated regions, with which polypeptides or other biomolecules interact to carry out transcription and translation. For eukaryotic cells, the control sequences will generally include a promoter and preferably an enhancer, for example, derived from immunoglobulin genes, SV40, cytomegalovirus, and a polyadenylation

sequence, and may include splice donor and acceptor sequences. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. When referring to Deglycating Enzyme, a promoter other than that naturally associated with the Deglycating Enzyme coding sequence can be referred to as a "heterologous" promoter.

A "deletion" refers to a change in an amino acid or nucleotide sequence due to the absence of one or more amino acid residues or nucleotides. The terms "insertion" or "addition" refer to changes in an amino acid or nucleotide sequence resulting in the addition of one or more amino acid residues or nucleotides, respectively, to a molecule or representation thereof, as compared to a reference sequence, for example, the sequence found in the naturally occurring molecule. A "substitution" refers to the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively. Amino acid sequence deletions range from about 1 to 30 residues, more preferably about 1 to 10 residues, and typically are contiguous. Deletions, may be introduced into regions not directly involved in the catalytic domain. Amino acid insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intra-sequence insertions of single or multiple amino acid residues. Intra-sequence insertions (*i.e.*, insertions within a Deglycating Enzyme amino acid sequence) may range generally from about 1 to about 10 residues, more preferably 1 to 5 residues, most preferably 1 to 3 residues. Examples of terminal insertions include Deglycating Enzyme polypeptides with an N-terminal methionyl residue, an artifact of its direct expression in bacterial recombinant cell culture, and fusion of a heterologous N-terminal signal sequence of the N-terminus of a Deglycating Enzyme molecule to facilitate the secretion of a mature a Deglycating Enzyme from recombinant host cells. Such signal sequences will generally be obtained from, and thus homologous to the intended host cells species. Suitable sequences include STII or lpp for E. coli, alpha factor for yeast, and viral signals such as herpes gD for mammalian cells.

The term "derivative" refers to a chemical modification of a polypeptide, polynucleotide, or other molecule. In the context of this invention, a "derivative polypeptide", for example, one modified by glycosylation, pegylation, or any similar process, retains one or more Deglycating Enzyme activities. For example, the term

"derivative" of Deglycating Enzyme refers to Deglycating Enzyme proteins, variants, or fragments that have been chemically modified, as, for example, by addition of one or more polyethylene glycol molecules, sugars, phosphates, and/or other such molecules, where the molecule or molecules are not naturally attached to wild-type Deglycating Enzyme polypeptides.

A "derivatized" polynucleotide, oligonucleotide, or nucleic acid generally refers to oligo- and polynucleotides that comprise a derivatized substituent. In some embodiments, the substituent is substantially non-interfering with respect to hybridization to complementary polynucleotides. Derivatized oligo- or polynucleotides that have been modified with appended chemical substituents (for example, by modification of an already synthesized oligo- or poly-nucleotide, or by incorporation of a modified base or backbone analog during synthesis) may be introduced into a metabolically active eukaryotic cell to hybridize with a Deglycating Enzyme DNA, RNA, or protein where they produce an alteration or chemical modification to a local DNA, RNA, or protein. Alternatively, a derivatized oligo or polynucleotides may interact with and alter Deglycating Enzyme polypeptides, or proteins that interact with Deglycating Enzyme DNA or Deglycating Enzyme gene products, or alter or modulate expression or function of Deglycating Enzyme DNA, RNA or protein. Illustrative attached chemical substituents include: europium (III) texaphyrin, cross-linking agents, psoralen, metal chelates (for example, iron/EDTA chelate for iron catalyzed cleavage), topoisomerases, endonucleases, exonucleases, ligases, phosphodiesterases, photodynamic porphyrins, chemotherapeutic drugs (for example, adriamycin, doxirubicin), intercalating agents, base-modification agents, immunoglobulin chains, and oligonucleotides. Iron/EDTA chelates are chemical substituents often used where local cleavage of a nucleic acid sequence is desired (Hertzberg *et al.*, 1982, *J. Am. Chem. Soc.* 104: 313; Hertzberg and Dervan, 1984, *Biochemistry* 23: 3934; Taylor *et al.*, 1984, *Tetrahedron* 40: 457; Dervan, 1986, *Science* 232: 464). Illustrative attachment chemistries include: direct linkage, for example, via an appended reactive amino group (Corey and Schultz, 1988, *Science* 238: 1401, which is incorporated herein by reference) and other direct linkage chemistries, although streptavidin/biotin and digoxigenin/anti-digoxigenin antibody linkage methods can also be used. Examples of methods for linking chemical substituents are provided in U.S. Patents 5,135,720, 5,093,245, and 5,055,556, which are incorporated herein by

reference. Other linkage chemistries may be used at the discretion of the practitioner.

A "Deglycating Enzyme" refers to a protein, preferably a substantially purified protein, having one or more Deglycating Enzyme activities. While Deglycating Enzymes for use in practicing the present invention may be obtained from any species, particularly preferred are Deglycating Enzymes isolated or derived from mammalian species (for example, bovine, canine, equine, feline, murine, ovine, porcine, equine), most preferably from the human species. In addition, Deglycating Enzymes may be obtained from any source, be it a natural, synthetic, semi-synthetic, or recombinant source. As indicated herein, the present invention also concerns Deglycating Enzyme fragments, analogs, variants, and derivatives, and polynucleotides encoding the same. Variants may be a naturally occurring allelic variants or non-naturally occurring variants, and include deletion variants, substitution variants, and addition or insertion variants. As known in the art and set forth herein, an allelic variant is an alternate form of a polynucleotide sequence that may have a substitution, deletion, or addition of one or more nucleotides. Variants also include such naturally occurring variants as splice variants. "Deglycating Enzyme" encompasses, but is not limited to, an enzyme which is encoded by SEQ ID NO:1 and and analogs thereof. The term "Deglycating Enzyme" also encompasses bioactive or functional fragments or portions of a Deglycating Enzyme having one or more desired Deglycating Enzyme activities, for example, kinase activity. Fragments or portions of a Deglycating Enzyme without activity may also be used, for example, for the generation of antibodies or for use in research.

The term "Deglycating Enzyme" also encompasses analogs of the sequences in SEQ ID NO:1 and fragments thereof. "Deglycating Enzyme" and its analogs also encompass functional and non-functional variants. Generally, Deglycating Enzymes exhibit deglycating activity and/or kinase activity, for example. A Deglycating Enzyme and its analogs are capable of interacting directly or indirectly with low molecular weight amines and amino acids including fructosamines, NEGs, AGEs and other sugar amines and/or glycoproteins or other sugar-bearing proteins and facilitating or effecting their degradation, for example, by phosphorylation. In another aspect, Deglycating Enzyme and its analogs are capable of generating oxidative damage that is associated with the degradation of low molecular weight amines and amino acids including

fructosamines, NEGs, AGEs, other sugar amines and/or glycoproteins or other sugar-bearing proteins.

"Deglycating activity" refers to the actions exhibited by Deglycating Enzyme. Examples of deglycating activities include but are not limited to activities described or referenced herein, including for example: phosphorylation; phosphorylation of sugars on glycoproteins or other sugar-bearing proteins; removal of fructosyl moieties from proteins; binding to low molecular weight amines and amino acids including fructosamines, NEGs, AGEs, other sugar amines and/or glycoproteins or other sugar-bearing proteins; binding to low molecular weight amines and amino acids including fructosamines, NEGs, and other sugar amines and facilitating or effecting their degradation; binding to low molecular weight amines and amino acids including fructosamines, NEGs, AGEs, other sugar amines and/or glycoproteins or other sugar-bearing proteins and facilitating or effecting their degradation to produce reactive oxygen species that may cause damage to tissues, cells, and cellular components.

As used herein, a "detectable label" has the ordinary meaning in the art and refers to an atom (for example, radionuclide), molecule (for example, fluorescein), or complex, that is or can be used to detect (for example, due to a physical, chemical or optical property), indicate the presence of a molecule or to enable binding of another molecule to which it is covalently bound or otherwise associated. The term "label" also refers to covalently bound or otherwise associated molecules (for example, a biomolecule such as an enzyme) that acts on a substrate to produce a detectable atom, molecule or complex. Detectable labels suitable for use in the present invention include, for example, any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical, chemical, or other means.

As used herein, "DNA" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides.

An "effective amount" (in the context of treatment or prophylaxis) is an amount sufficient to effect one ore more beneficial or desired results, for example, by carrying out or antagonizing one or more activities of a Deglycation Enzyme. This encompasses clinical results or delaying the onset of a condition or a disease state (or one or more

symptoms of either) associated with an abnormal amount of production or degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins, for example. An effective amount can be administered in one or more administrations.

The term "epitope" has its ordinary meaning of a site on an antigen or antigenic molecule recognized by an antibody. Epitopes may be segments of amino acids, including segments that represent a small portion of a whole protein or polypeptide. Epitopes may be conformational (*i.e.*, discontinuous). That is, they may be formed from amino acids encoded by noncontiguous parts of a primary sequence that have been juxtaposed by protein folding.

"FAOORase enzyme" refers to an enzyme belonging in the class designated as EC1.5.3. See, Enzyme Nomenclature, Recommendations of the Nomenclature Committee of the International Union of Biochemistry, Academic Press, London pp.19-22 (1979). Enzymes of this class react with low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs to degrade them, which results in production of undesired reactive oxygen species. In addition, FAOORase enzymes exhibit activity which can result in the deglycation of sugars from proteins that have been modified due to non-enzymatic glycation. Examples of substrates for this activity include advanced glycation endproducts (AGE's). The process of non-enzymatic glycation involves Schiff base formation →Amadori compounds →→ advanced glycation endproducts (AGEs). In addition to "FAOORase enzymes", another class of enzymes which may cause deglycation through a kinase activity has been characterized. This class is fructosamine-3-kinases. See, for example, Delpierre G., Rider MH., Collard F. *et al. Diabetes 49:1627-1634 (2000),* and Delpierre G., Vanstapel F., Stroobant V. *et al. Biochem. J. 352:835-839 (2000).*

As used herein, the terms "functional fragment" or "functional analog" refer to polypeptides which are generally homologous to a Deglycating Enzyme and have one or more Deglycating Enzyme activities. Thus, the phrases "functional analog" or "functional fragment" of a native polypeptide refer to compounds having qualitative biological activity in common with a native polypeptide. For example, a functional fragment or analog of a Deglycating Enzyme is a compound that has a one or more qualitative biological activities in common with Deglycating Enzyme, such as one that

can modulate or assist in modulating glycoproteins or other sugar-bearing proteins, for example by phosphorylation or deglycation. "Functional fragments" include, but are not limited to, peptide fragments of the native polypeptide from any animal species (including humans), and derivatives of native (human and non-human) polypeptides and their fragments, provided that they are able to qualitatively effect one or more activities of the the full-length polypeptide. The term "analog" includes an amino acid sequence and its glycosylation variants which also share functionality similar to a full-length active Deglycating Enzyme.

A "functional site" of a protein refers to any site in a protein that has a function. Representative examples include active sites (*i.e.*, those sites in catalytic proteins where catalysis occurs), protein-protein interaction sites, sites for chemical modification (for example, glycosylation and phosphorylation sites), and ligand binding sites. Ligand binding sites include metal ion binding sites, co-factor binding sites, antigen binding sites, substrate channels and tunnels, and substrate binding sites. In an enzyme, a ligand binding site that is a substrate binding site may also be an active site, or overlap with an active site. As used herein, the "biochemical function" of a functional site refers to the function carried out by the site in a naturally occurring protein that possesses the corresponding function. For example, the biochemical function of an active site refers to the specific catalytic activity of the site, whereas the biochemical function of a substrate binding site is the binding of the particular substrate.

The term "fusion protein," refers to a composite polypeptide, *i.e.*, a single contiguous amino acid sequence, made up of two (or more) distinct, polypeptides that fused or otherwise linked together, directly or indirectly, in a single amino acid sequence. Thus, for example, a fusion protein may include a single amino acid sequence that contains two entirely distinct amino acid sequences or two similar or identical polypeptide sequences that are not normally found together in the same configuration in a single amino acid sequence found in nature. Fusion proteins may generally be prepared using either recombinant nucleic acid methods, *i.e.*, as a result of transcription and translation of a recombinant gene fusion product, which fusion comprises a segment encoding a polypeptide of the invention and a segment encoding a heterologous polypeptide, or by chemical synthesis methods well known in the art. Generally, a "fusion polypeptide" is a polypeptide comprising regions in a different position than

occurs in nature. The regions may normally exist in separate proteins and are brought together in the fusion polypeptide, or they may normally exist in the same protein but are placed in a new arrangement in the fusion polypeptide. A fusion polypeptide may also arise from polymeric forms, whether linear or branched, for example, the binding region of Deglycation Enzyme fused to a marker which may be used for selection, purification, or visualization purposes. The invention includes fusion proteins comprising a Deglycating Enzyme, or an analog, fragment, variant, derivative or homologue thereof, for example.

The term "gene product" refers to an RNA molecule transcribed from a gene, or a polypeptide encoded by the gene or translated from the RNA.

The term "high affinity" for an IgG antibody, as used herein, refers to an association constant (Ka) of at least about 10^6M^{-1}, preferably at least about 10^8M^{-1}, more preferably at least about 10^9M^{-1} or greater, for example, up to $10^{12}M^{-1}$ or greater. However, "high affinity" binding can vary for other antibody isotypes. The invention includes antibodies against proteins comprising, consisting essentially of, or consisting of a Deglycating Enzyme, or an analog, fragment, variant, derivative or homologue thereof, for example.

As used herein, the term "homologous" refers to similarity of sequences (either protein or nucleic acid). Homologous polypeptides can contain stretches of sequences which are identical to a portion of Deglycating Enzyme. Alternatively, the sequences can still be homologous without containing stretches of sequences which are identical to a portion of Deglycating Enzyme. Sequences which are homologous can be determined by alignment against full length Deglycating Enzyme or a portion of Deglycating Enzyme. Typical homologous proteins or peptides will have from 25-100% homology, to 50-100% homology with the amino acid sequence of the Deglycating Enzyme. Homology measures will be at least about 35%, generally at least 40%, more generally at least 45%, and more typically sequences which are homologous to Deglycating Enzyme are at least about 50% identical to Deglycating Enzyme, more typically at least about 60% identical, more typically at least about 70% identical, more typically at least about 75% identical, more typically at least about 80% identical, more typically at least about 85% identical, more typically at least about 90% identical, and more typically at least

about 95% identical. The invention includes polypeptide that are homologous to a Deglycating Enzyme.

A "homologue," in the context of a polypeptide or nucleic acid molecule, implies an evolutionary relationship, *i.e.*, descendent from a common ancestor. The invention includes homologues of Deglycating Enzymes.

A "host cell" or "recombinant host cell" includes an individual cell or cell culture which can be or has been a recipient for vector(s) or for incorporation of polynucleotides and/or proteins. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in genomic of total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation. A host cell includes cells transfected *in vivo* with a polynucleotide(s) of this invention, including for example a cell that contains a vector, for example, a cloning vector or an expression vector, or a cell that has otherwise been manipulated by recombinant techniques to express a protein of interest. As noted herein, the invention includes recombinant host cells and host cells that express, for example, Deglycating Enzymes and fragments, analogs, derivatives, variants and homologues thereof.

"Hybridization" refers to any process by which a single-stranded nucleic acid molecule, portion thereof, or single-stranded region of an otherwise double-stranded nucleic acid molecule binds through base pairing with a complementary single-stranded nucleic acid molecule, portion thereof, or single-stranded region of an otherwise double-stranded nucleic acid molecule. Hybridization may be performed where both nucleic acid molecules are in solution, or between one nucleic acid molecule in solution and another nucleic acid molecule immobilized on a solid support (for example, paper, membranes, filters, chips, pins, glass slides, or any other appropriate substrate to nucleic acids can be fixed).

The terms "immunogen" and "immunogenic" have their ordinary meaning in the art, *i.e.*, an immunogen is a molecule, such as a polypeptide or other antigen, that can elicit an adaptive immune response upon introduction into a person or an animal. For example, the term "immunogen" includes a chemical entity that elicits a humoral immune response when injected into an animal. Immunogens may have both B cell epitopes and T cell epitopes. The invention includes relevant or useful immunogens for the production of antibodies, for example.

"In conjunction with" refers to administration of one treatment modality in addition to another treatment modality, such as administration of an agent described herein in addition to administration of another agent to the same individual. As such, "in conjunction with" refers to administration of one treatment modality before, during or after delivery of the other treatment modality to the individual. For example, it is possible to use this treatment in conjunction with one or more of the following anti-diabetic or hypoglycemic agents: insulin, sulfonylureas, glitazones, biguanidines (*e.g.*, metformin), or pramlintide. Other classes of agents with which Deglycating Enzyme therapy or anti-Deglycating Enzyme therapy may be used include but are not limited to: anti-hypertensives, lipid lowering agents, anti-arrhythmics, calcium channel blockers, and anti-heart failure medication.

An "individual" is a generally a vertebrate, preferably a mammal, and more preferably a human. Mammals include, but are not limited to, farm animals, sport animals, pets, primates, mice and rats.

An "isolated" molecule (for example, a polypeptide or polynucleotide) refers to a molecule that is present outside of some or all of its original environment or has been removed from some or all of its original environment (for example, the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system (for example, proteins, lipids, carbohydrates, nucleic acids), is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment. The invention includes any of the polypeptides and polynucleotides referred to herein that are isolated.

The term "modulate" refers to a change in the biochemical activity. For example, modulation may involve an increase or a decrease in catalytic rate, substrate binding characteristics, *etc.* Modulation may occur, for example, by covalent or non-covalent interaction with the protein, and can involve an increase or decrease in biochemical activity. A "modulator" refers to a compound that causes a change, *i.e.*, an increase or decrease, in activity of a protein, and, for example, is typically a ligand, either peptidic, polypeptidic, or small molecule (for example, an agonist or antagonist). A modulator

may act directly, for example, by interacting with a protein to cause an increase or decrease in activity. A modulator may also act indirectly, for example, by interfering with, *i.e.*, antagonizing or blocking, the action of another molecule that causes an increase or decrease in activity of the protein. The terms "modulator" and "modulation" of Deglycating Enzyme activity, as used herein in its various forms, is intended to encompass antagonism, agonism, partial antagonism and/or partial agonism of the activity associated with the Deglycating Enzyme protein or gene. In various embodiments, "modulators" may inhibit or stimulate Deglycating Enzyme expression or one ore more Deglycating Enzyme activities. Such modulators include small molecules agonists and antagonists of Deglycating Enzyme function or expression, antisense molecules, ribozymes, triplex molecules, and RNAi polynucleotides, gene therapy methods, and others.

"Naturally occurring" refers to an endogenous chemical moiety, such as a carbohydrate, polynucleotide or polypeptide sequence, *i.e.*, one found in nature. Processing of naturally occurring moieties can occur in one or more steps, and these terms encompass all stages of processing. Conversely, a "non-naturally occurring" moiety refers to all other moieties, *e.g.*, ones which do not occur in nature, such as recombinant polynucleotide sequences and non-naturally occurring carbohydrates.

"Non-enzymatic glycation products" and "NEGs" are used interchangeably throughout and refers to substrates which have been modified by the addition of a fructosyl moiety. NEGs are the precursors of advanced glycation endproducts (AGEs). Collectively, fructosyl-modified proteins and AGEs are encompassed by the term "non-enzymatic glycation products (NEGs)".

The phrases "nucleic acid", "nucleic acid molecule", and the like refer to a nucleotide, oligonucleotide, polynucleotide, or any fragment thereof. These phrases also refer to single-stranded or double-stranded DNA and/or RNA of cellular or synthetic origin. In this context, "fragments" refer to those nucleic acid molecules that, when translated, produce polypeptides retaining some functional characteristic, for example, antigenicity or a structural domain of a naturally occurring polypeptide. Unless specifically limited, the disclosure of a polynucleotide sequence is also intended to refer to the complementary sequence. As used herein, the term "polynucleotide" includes oligonucleotides.

The term "oligonucleotide" generally refers to a nucleic acid sequence of at least about 6 nucleotides to about 100 nucleotides, preferably about 15 to about 50 nucleotides, and most preferably about 20 to 40 nucleotides, which can be used, for example, as primers or probes in PCR amplification or in a hybridization assay. As used herein, the term "oligonucleotide" includes "amplimers," "primers," "oligomers," and "probes," as these terms are commonly used in the art. The invention includes isolated oligonucleotides coding for any of the polypeptides referred to herein.

The terms "operably associated" and "operably linked" refer to functionally related nucleic acid molecules. For example, a promoter is operably associated with or operably linked to a coding sequence if the promoter assists in control of transcription and/or translation of the encoded polypeptide in an appropriate host cell or other expression system. While operably associated or operably linked nucleic acid molecules can be contiguous and in the same reading frame, certain genetic elements need not be contiguously linked to the nucleic acid encoding the polypeptide(s) to be expressed. For example, enhancers need not be located in close proximity to the coding sequences whose transcription they enhance. The invention includes oligonucleotides coding for any of the polypeptides referred to herein that are operably linked.

The terms "peptidomimetic" and "mimetic" refer to a synthetic chemical compound that may have substantially the same structural and functional characteristics of the Deglycating Enzymes of the invention and that mimic a Deglycating Enzyme structure and/or activity, at least in part and to some degree. Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics" (Fauchere, *J. Adv. Drug Res.* 15:29 (1986); Veber and Freidinger *TINS* p. 392 (1985); and Evans et al. *J. Med. Chem.* 30:1229 (1987)). Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent or enhanced therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (*i.e.*, a polypeptide that has a biological or pharmacological activity), such as a Deglycating Enzyme, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of, for examplefor example, -CH2NH-, -CH2S-, -CH2-CH2-, -CH=CH- (cis and trans), -COCH2-, -CH(OH)CH2-, and -CH2SO-.

The mimetic can be either entirely composed of synthetic, non-natural analogs of amino acids, or, is a chimeric molecule of partly natural peptide amino acids and partly non-natural analogs of amino acids. The mimetic can also incorporate any amount of natural amino acid conservative substitutions as long as such substitutions also do not substantially alter the mimetic's structure and/or activity. For example, a mimetic composition is within the scope of the invention if it is capable of carrying out the binding or one or more other biological activities of a Deglycating Enzyms, for example, enzymatic activity. The invention includes peptidomimetics and mimetics of any of the polypeptides referred to herein.

The phrase "percent (%) identity" refers to the percentage of sequence similarity found in a comparison of two or more amino acid sequences. Percent identity can be determined electronically using any suitable software. Likewise, "similarity" between two polypeptides (or one or more portions of either or both of them) is determined by comparing the amino acid sequence of one polypeptide to the amino acid sequence of a second polypeptide. Any suitable algorithm useful for such comparisons can be adapted for application in the context of the invention.

By "pharmaceutically acceptable" it is meant, for example, a carrier, diluent or excipient that is compatible with the other ingredients of the formulation and generally safe for administration to a recipient thereof. The invention includes, for example, polypeptides and peptidomimetics and mimetics of any of the polypeptides referred to herein in a pharmaceutically acceptable formulation.

The term "polypeptide" is used interchangeably herein with the term "protein," and refers to a polymer composed of amino acid residues linked by amide linkages, including synthetic, naturally-occurring and non-naturally occurring analogs thereof (amino acids and linkages). Peptides are examples of polypeptides.

A "polynucleotide" means a plurality of nucleotides. Thus, the terms "nucleotide sequence" or "nucleic acid" or "polynucleotide" or "oligonculeotide" are used interchangeably and refer to a heteropolymer of nucleotides or the sequence of these nucleotides. These phrases also refer to DNA or RNA of genomic or synthetic origin which may be single-stranded or double-stranded and may represent the sense or the antisense strand, to peptide nucleic acid (PNA) or to any DNA-like or RNA-like material. It is contemplated that where the polynucleotide is RNA, the T (thymine) in

the sequences provided herein is substituted with U (uracil). A polynucleotide that encodes a Deglycating Enzyme, a Deglycating Enzyme fragment, a Deglycating Enzyme variant, a Deglycating Enzyme analog, or a Deglycating Enzyme homologue refers to a polynucleotide encoding, for example: the mature form of a Deglycating Enzyme found in nature; the mature form of a Deglycating Enzyme found in nature and additional coding sequence, for example, a leader or signal sequence or a proprotein sequence; either of the foregoing and non-coding sequences (for example, introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature form of the polypeptide found in nature); and fragments, variants, analogs and homologues of the mature form of a Deglycating Enzyme found in nature. Thus, the term "Deglycating Enzyme-encoding polynucleotide" and the like encompass polynucleotides that include only a coding sequence for a desired Deglycating Enzyme, fragment, variant, analog or homologue as well as a polynucleotide that includes additional coding and/or non-coding sequences, for example. Thus, the terms "polynucleotide" and "nucleic acid", used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. These terms include a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. It is understood that the double stranded polynucleotide sequences described herein also include the modifications described herein. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be a oligodeoxynucleoside phosphoramidate (P-NH2) or a mixed phosphoramidate-phosphodiester oligomer. A phosphorothioate linkage can be used in place of a phosphodiester linkage. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand *de novo* using a DNA polymerase with an appropriate primer. The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides,

branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide is said to "encode" a polypeptide if, in its native state or when manipulated by methods well known to those skilled in the art, it can be transcribed and/or translated to produce the polypeptide or a fragment thereof. For purposes of this invention, and to avoid cumbersome referrals to complementary strands, the anti-sense (or complementary) strand of such a polynucleotide is also said to encode the sequence; that is, a polynucleotide sequence that "encodes" a polypeptide includes both the conventional coding strand and the complementary sequence (or strand).

As used herein, a "probe," when used in the context of polynucleotides and antibodies, refers to a molecule that specifically binds another molecule. One example of a probe is a "nucleic acid probe," which can be a DNA, RNA, or other polynucleotide. Where a specific sequence for a nucleic acid probe is given, it is understood that the complementary strand is also identified and included. The complementary strand will work equally well in situations where the target is a double-stranded nucleic acid that specifically binds (for example, anneals or hybridizes) to a substantially complementary nucleic acid. Another example of a probe is an "antibody probe" that specifically binds to a corresponding antigen or epitope. The invention includes probes for Deglycating Enzymes and homologues, for example.

In general, the term "protein" refers to any polymer of two or more individual amino acids (whether or not naturally occurring) linked via peptide bonds, as occur when the carboxyl carbon atom of the carboxylic acid group bonded to the α-carbon of one amino acid (or amino acid residue) becomes covalently bound to the amino nitrogen atom of the amino group bonded to the α-carbon of an adjacent amino acid. These peptide bond linkages, and the atoms comprising them (*i.e.*, α-carbon atoms, carboxyl carbon atoms (and their substituent oxygen atoms), and amino nitrogen atoms (and their substituent hydrogen atoms)) form the "polypeptide backbone" of the protein. In addition, as used herein, the term "protein" is understood to include the terms "polypeptide" and "peptide" (which, at times, may be used interchangeably herein). Similarly, protein fragments, analogs, derivatives, and variants are may be referred to herein as "proteins," and shall be deemed to be a "protein" unless otherwise indicated.

The term "fragment" of a protein refers to a polypeptide comprising fewer than all of the amino acid residues of the protein. As will be appreciated, a "fragment" of a protein may be a form of the protein truncated at the amino terminus, the carboxy terminus, and/or internally (such as by natural splicing), and may also be variant and/or derivative. A "domain" of a protein is also a fragment, and comprises the amino acid residues of the protein required to confer biochemical activity corresponding to naturally occurring protein. A "variant" or "analog" refers to a protein altered by one or more amino acids in relation to a reference protein (for example, a naturally occurring form of the protein), for example, by one or more amino acid sequence substitutions, deletions, and/or insertions. A variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties (for example, replacement of leucine with isoleucine). Alternatively, a variant may one or more have "non-conservative" changes (for example, replacement of glycine with tryptophan). Other variations include amino acid deletions or insertions, or both. Such variants can be prepared from corresponding nucleic acid molecule variants, which have a nucleotide sequence that varies accordingly from the nucleotide sequences, for example, for wild-type Deglycating Enzyme polypeptides. Unless otherwise indicated, a protein's amino acid sequence (*i.e.*, its "primary structure" or "primary sequence") will be written from amino-terminus to carboxy-terminus. In non-biological systems (for example, those employing solid state synthesis), the primary structure of a protein (which also includes disulfide (cysteine) bond locations) can be determined by the user. In addition to primary structure, proteins also have secondary, tertiary, and, in multisubunit proteins, quaternary structure. "Secondary structure" refers to local conformation of the protein chain, with reference to the covalently linked atoms of the peptide bonds and α-carbon linkages that string the amino acid residues of the protein together. Representative examples of secondary structures include α helices, parallel and anti-parallel β structures, and structural motifs such as helix-turn-helix, β-α-β, the leucine zipper, the zinc finger, the β-barrel, and the immunoglobulin fold. "Tertiary structure" concerns the three-dimensional structure of a protein, including the spatial relationships of amino acid side chains and atoms, and the geometric relationships of different regions of the protein. "Quaternary structure" refers to the structure and non-covalent association of different

polypeptide subunits in a multisubunit protein. As noted herein, the invention includes Deglycating Enzymes and fragments, analogs, derivatives, variants and homologues thereof.

As used herein, a "pure" Deglycating Enzyme, analog, fragment, variant or derivative refers to a preparation or composition which is comprised of at least 95% of said Deglycating Enzyme, analog, fragment, variant or derivative and more preferably at least about 99% of said Deglycating Enzyme, analog, fragment, variant or derivative. As used herein, "substantially pure" or "essentially pure" Deglycating Enzyme or Deglycating Enzyme analog, variant, fragment, or derivative refers to a composition which is comprised of at least about 50% of said Deglycating Enzyme, analog, fragment, variant or derivative, preferably at least about 65% of said Deglycating Enzyme, more preferably at least about 75% of said Deglycating Enzyme, more preferably at least about 85% of said Deglycating Enzyme, analog, fragment, variant or derivative, and still more preferably at least about 90% to about 95%.

The term "recombinant" refers to a polynucleotide synthesized or otherwise manipulated *in vitro* (for example, "recombinant polynucleotide"), to methods of using recombinant polynucleotides to produce gene products in cells or other biological systems, or to a polypeptide ("recombinant protein") encoded by a recombinant polynucleotide. Thus, a "recombinant" polynucleotide is defined either by its method of production or its structure. In reference to its method of production, the process refers to use of recombinant nucleic acid techniques, for example, involving human intervention in the nucleotide sequence, typically selection or production. Alternatively, it can be a polynucleotide made by generating a sequence comprising a fusion of two or more fragments that are not naturally contiguous to each other. Thus, for example, products made by transforming cells with any non-naturally occurring vector is encompassed, as are polynucleotides comprising sequence derived using any synthetic oligonucleotide process. Similarly, a "recombinant" polypeptide is one expressed from a recombinant polynucleotide. As noted herein, the invention includes recombinant Deglycating Enzymes and fragments, analogs, derivatives, variants and homologues thereof, as well as recombinant polynucleotides and vectors (including cloning and expression vectors) coding for Deglycating Enzymes and fragments, analogs, derivatives, variants and homologues thereof, for example.

The phrase "selectively hybridizing to" refers to a polynucleotide probe that hybridizes, duplexes or binds to a particular target DNA or RNA sequence at a desired level of reactivity or nonreactivity with non-target molecules when the target sequences are present in a preparation of total cellular DNA or RNA. The invention includes probes that selectively hybridize to, for example, polynucleotides coding for Deglycating Enzymes and fragments, analogs, derivatives, variants and homologues thereof.

"Transformation" or "transfection" refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion, for example, lipofection, transduction, infection or electroporation. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host cell genome.

As used herein, "treatment" is an approach for obtaining one or more beneficial or desired results, including and preferably clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, one or more of the following: amelioration or prevention, in whole or in part, of one or more symptoms or aspects of the condition or disease to be treated or prevented; protein deglycation; removal of sugar molecules from proteins; phosphorylation of proteins; phosphorylation of sugar molecules on proteins; lowered or enhanced rates of degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins; lowered or enhanced amounts of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs degradation; decrease in the amounts of reactive oxygen species generated during the degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs; palliating damage resulting from Deglycating Enzyme activities in an individual; amelioration of damage associated with reactive oxygen species; amelioration of cardiovascular damage associated with excessive production of reactive oxygen species; and amelioration of a disease state associated with undesired degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs, for example. "Palliating damage resulting from Deglycating Enzyme activities in an individual" means lessening the extent and/or time course of undesirable clinical manifestations, for example, manifestations of oxidative damage that may result

from Deglycating Enzyme activities in an individual. Individuals or populations of individuals may be treated with an agent that antagonizes the activity of Deglycating Enzyme in accordance with the invention. "Reducing severity of a symptom" or "ameliorating a symptom" of damage associated with (or prevented by) one or more Deglycating Enzyme activities in an individual means a lessening or improvement of one or more symptoms as compared to not administering an agent that antagonizes (or carries out) one or more activities of a Deglycating Enzyme. Such symptoms include, but are not limited to, generation of reactive oxygen species, damage to various tissues (*e.g.*, heart, arteries, kidney, liver, eyes, nerves, *etc.*) resulting from reactive oxygen species or glycation of proteins, macrovascular or microvascular damage, and cardiomyopathy. As used herein, "complications associated with diabetes" refers to physiological and/or clinical events which occur in an individual with diabetes. Non-limiting example include: macrovascular and microvascular damage, cardiomyopathy, renal dysfunction, renal failure, blindness, gangene-related amputations, coronary heart disease, and ischemic brain damage. As used herein, "delaying" development of diabetes or disease states means to defer, hinder, slow, retard, stabilize, and/or postpone development of various symptoms or pathologies associated respectively with diabetes and the disease states. Symptoms which are associated with, for example, excessive amounts of production or degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins are described herein. Symptoms can also include complications associated with diabetes, as referenced herein. This delay can be of varying lengths of time, depending on the history of the individual being treated. As is evident to one skilled in the art, a sufficient or significant delay can, in effect, encompass prevention, in that the individual does not develop (or develop further) pathologies and/or symptoms associated with, for example, excessive amounts of production or degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins. A method that "delays" development of disease states associated with, for example, excessive amount of production or degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins is a method that reduces probability of development of the

pathologies or symptoms associated with, for example, excessive amount of production or degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins in a given time frame and/or reduces extent of the disease in a given time frame, when compared to not using the method. Such comparisons may be based on clinical studies using a statistically significant number of subjects. "Development" of a disease state associated with, for example, excessive amount of degradation of low molecular weight amines and amino acids, including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins, means the onset and or progression of a disease state (*e.g.*, macrovascular and microvascular damage) within an individual. Macrovascular and microvascular damage development can be detected using standard clinical techniques known to a skilled artisan. "Development" also refers to disease progression that may be initially undetectable. For purposes of this invention, progression refers to the biological course of the disease state. Such a biogical course may include protein glycation, the generation of reactive oxygen species, and the resulting tissue damage. "Development" includes occurrence, recurrence, and onset. As used herein "onset" or "occurrence" of a disease state associated with, for example, excessive amount of degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins includes initial onset and and/or recurrence. "Receiving treatment" includes initial treatment and/or continuing treatment.

Methods of the invention

With respect to all composition of matter and methods described herein, reference to compositions such as agents which antagonize Deglycation Enzyme activity also include compositions comprising one or more of these substances. These compositions may further comprise suitable excipients, such as pharmaceutically acceptable excipients including buffers, which are well known in the art.

Isolation and Characterization of Deglycation Enzyme

In one broad aspect, the present invention is directed to a Deglycation Enzyme. Deglycation Enzymes have phosphorylation or kinase activity and can directly or indirectly modulate or degrade low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-

bearing protein. They may be characterized as a member of the class of proteins that include FAOORases, including but not limited to those that fall in enzyme class EC1.5.3. The amino acid sequence of a Deglycation Enzyme is as follows:

MEELLRRELG CSSVRATGHS GGGCISQGRS YDTDQGRVFV KVNPKAEARR 50
MFEGEMASLT AILKTNTVKV PKPIKVLDAP GGGSVLVMEH MDMRHLSSHA 100
AKLGAQLADL HLDNKKLGEM RLKEAGTVGR GGGQEERPFV ARFGFDVVTC 150
CGYLPQVNDW QEDWVVFYAR QRIQPQMDMV EKESGDREAL QLWSALQLKI 200
PDLFRDLEII PALLHGDLWG GNVAEDSSGP VIFDPASFYG HSEYELAIAG 250
MFGGFSSSFY SAYHGKIPKA PGFEKRLRLY QLFHYLNHWN HFGSGYRGSS 300
LNIMRNLVK 309(SEQ ID NO:1).

In one aspect, the protein comprises a variant of SEQ ID NO:1 comprising a substitution of isoleucine to valine in the amino acid at position 62 in SEQ ID NO:1. In another aspect, the protein comprises a variant of SEQ ID NO:1 comprising a substitution of arginine to glutamine in the amino acid at position 278 in SEQ ID NO:1. In still another aspect, the protein comprises a variant of SEQ ID NO:1 comprising two amino acid substitutions, specifically a isoleucine to valine substitution at position 62 in the amino acid sequence and an arginine to glutamine substitution at position 278 in the amino acid sequence of SEQ ID NO:1. The protein comprising these amino acid substitutions can be encoded by a polynucleotide comprising single nucleotide changes for each amino acid substitution.

In another aspect, a polynucleotide encoding the protein of SEQ ID NO:1 comprises the following nucleic acid sequence:

1 gcggccgcgg cgggaacatg gaggagctgc tgaggcgcga gctgggctgc agctctgtca
61 gggccacggg ccactcgggg ggcgggtgca tcagccaggg ccggagctac gacacggatc
121 aaggacgagt gttcgtgaaa gtgaacccca aggcggaggc cagaagaatg tttgaaggtg
181 agatggcaag tttaactgcc atcctgaaaa caaacacggt gaaagtgccc aagcccatca
241 aggttctgga tgccccaggc ggcgggagcg tgctggtgat ggagcacatg gacatgaggc
301 atctgagcag tcatgctgca aagcttggag cccagctggc cgatttacac cttgataaca
361 agaagcttgg agagatgcgc ctgaaggagg cgggcacagt ggggagagga ggtgggcagg
421 aggaacggcc ctttgtggcc cggtttggat ttgacgtggt gacgtgctgt ggatacctcc
481 cccaggtgaa tgactggcag gaggactggg tcgtgttcta tgcccggcag cgcattcagc
541 cccagatgga catggtggag aaggagtctg gggacaggga ggccctccag ctttggtctg
601 ctctgcagtt aaagatccct gacctgttcc gtgacctgga gatcatccca gccttactcc
661 acggggacct ctggggtgga aacgtagcag aggattcctc tgggccggtg atttttgacc

```
721   cagcttcttt ctacggccac tcggaatatg agctggcaat agctggcatg tttgggggct
781   ttagcagctc cttttactcc gcctaccacg gcaaaatccc caaggcccca ggattcgaga
841   agcgccttcg gttgtatcag ctctttcact acttgaacca ctggaatcat tttggatcgg
901   ggtacagagg atcctccctg aacatcatga ggaatctggt caagtgagcg ggccttactc
961   tggaaggagg cctcagaggt ttctccacag tcctcttctg ggcaaattct tgtttcttca
1021  catgccggac tagcttaaga ccaatgcagt agcttatttc caagccttgc aaagtatata
1081  atatctaaga ggaaaggttt tgtcatccca gcgttgtcca ctttgtgggg ctttgtaggt
1141  agacggagcc acactacagg cagggtatga gcagagggat gtatggagtg tgggtgactc
1201  tgagcctcac tgctgctgca aggtggggaa actgtaagtg aacccctgtg ggtgcggggg
1261  agggtatccg gtgcgcaggg aggtggccag cgcccccggg cactgctgct cataggtacc
1321  tttccactgc ctcctccctg ctctcctgtg caggaatgtc tctgagctgt tcacgttgat
1381  gcttcttggt tggcaagact tgggtgtaga catgaaacca tcttactaaa agtgtcttaa
1441  aatgaccaat tccagaatca agcgtattcc gttttcttcc tgcatgatcc ctgggccctc
1501  ccgcaggctg agcaagtctg taaactgatt ctgggagaaa ccaagctgct ggccataggg
1561  tgtccttggg tacatccagg agtcttcatt gcttctgtta ttaccccgtc tcctctgcca
1621  ttttctacag cttgctgagt tgtcattcct ttgcaacatt aaaatacatg ctgaactc
```

(SEQ ID NO:2).

A polynucleotide encoding a Deglycating Enzyme was isolated by using primers designed from a full length cDNA sequence that was available through the NEDO human cDNA sequencing project (Takao Isogai, Helix Research Institute). This sequence encodes a protein expected to have a molecular weight of approximately 35kDa that displays approximately 66% sequence identity with a previously cloned human fructosamine-3-kinase. Primers for use in PCR amplification were designed specifically to be complementary to the gene sequence and to flank the open reading frame encoding Deglycating Enzyme. BLAST searches have also identified a number of microbial sequences encoding proteins sharing approximately 30% sequence identity with the human fructosamine-3-kinase, which is consistent with findings from other groups (Delpierre *et al.*, 2000). None of the fructosamine kinase sequences identified to date show any significant homology with any other kinase families. However, alignments of these sequences show a number of conserved residues that appear to correspond to conserved residues seen in both the serine/threonine kinases and the aminoglycoside kinases. These include the conserved lysine, glutamic acid, and aspartic acid residues at positions 41, 55, and 244, as well as the conserved DxxxN motif extending from residue 227 to 232 (Figure 5). Evidence from studies in the

aminoglycoside kinases suggest that these residues form an ATP binding site (Hon, W., *et al.*, *Cell* 89: 887-895 (1997)).

Without being bound by theory, it is believed that Deglycating Enzyme binds to and degrades or initiates the phosphorylation and/or degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins. One result of the phosphorylation and/or degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins by a Deglycating Enzyme may be the generation of deglycated proteins or proteins with reduced numbers of sugars. Another result of the phosphorylation and/or degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins by a Deglycating Enzyme may be the generation free radicals, such as reactive oxygen species. Other characterization of Deglycating Enzyme include but are not limited to expression pattern in tissues or cells, determination of structure, regulation of Deglycating Enzyme *in vivo*, measurement of deglycating activity, measurement of kinase activity, and measurement of oxidative stress and free radical formation.

To determine expression pattern of Deglycation Enzyme, the gene encoding Deglycation Enzyme can be expressed using commercially available expression vectors (*e.g.*, PET-17 and pMet) in bacterial (*e.g.*, *E. coli*) and/or eukaryotic (*e.g.*, *Saccharomyces cerevisiae*, *Pichia methanolica*) cellular systems. The resulting constructs are then transfected into cultured cells (*e.g.*, aortic endothelial cells Cos-7) to generate both transiently and stably transfected cells. Transfection techniques which can be used are standard protocols known in the art. Stable transfection involves the use of a selectable marker, such as neomycin resistance. Cellular production of the gene and gene product can be analyzed by Northern blotting, protein analysis by Western blotting, and/or direct sequence analysis. Cellular location can also be analyzed to determine whether the Deglycation Enzyme gene product is secreted, intracellular, or attached to the cell membrane by analyzing fractionated portions of the cells in which Deglycation Enzyme is expressed. Antibodies raised against the full length Deglycation Enzyme, a portion thereof, or from GST-fusions can be used to examine cellular location using

immunocytochemistry. Optionally, epitope-tagged constructs, such as through attachment of a flag or myc-epitope can be utilized. Deglycating Enzyme may also be characterized by its binding patterns to various human tissues using Northern blots.

To determine the structure of Deglycation Enzyme, Deglycation Enzyme gene can be expressed as a fusion protein in an appropriate vector construct (*e.g.*, as a fusion with Glutathione-S-transferase (GST) or maltose-binding protein). The resulting constructs can also be expressed in either bacterial or eukaryotic systems. Optionally, the Deglycation Enzyme gene can be modified using mutagenesis to include an epitope (*e.g.*, polyhistidine tag) on either the N-terminus or C-terminus. The epitope can be used to facilitate purification of the gene products (*e.g.*, glutathione beads or column chelation of the histidine-tag epitope). Once purified, the recombinant protein can be crystallized according to standard protocols (*e.g.*, hanging drop technique). Crystals can be then subjected to X-ray diffraction and subsequent analysis to determine the three dimensional structure of the protein. This information can be used in combination with other characterizations to identify the active site of Deglycation Enzyme, and serve as a basis for structure-based drug design.

To determine how Deglycation Enzyme is regulated, animal models of diabetes (*e.g.*, streptozocin-induced diabetes in rats; human amylin transgenic mice) can be utilized to analyze the expression patterns in both animal models of diabetes and non-diabetic animals or normal animals using Northern and Western blotting, and *in situ* hybridization techniques.

To measure glycation activity, a series of assays using a range of glycated substrates can be utilized. Examples of substrates which can be used include but are not limited to: synthetic fructosamine, 1-deoxy-l-morpholinofructose (DMF), glycated bovine, or human serum albumin. Activities of Deglycation Enzyme can be measured using purified glycated substrates from either bacterial or eukaryotic (*e.g.*, yeast) sources, and on transfected cellular systems. For transfected cellular systems, substrates can be overlayed on the cultured cells and incubated under defined culture conditions. As substrate phosphorylation may be an intermediate step contributing to the deglycation process, enzyme-catalyzed phosphorylation of these substrates can be measured following purification by anion-exchange chromatography or gel extrusion chromatography. Phosphorylation can be measured, for example, by MALDI-TOF mass

spectrometry, NMR, and in the case of glycated protein substrates, two-dimensional gel electrophoresis. Phosphorylation of fructose, a possible substrate for this enzyme, can also be measured using MALDI-TOF mass spectrometry.

To determine oxidative stress and free radical formation, cells (*e.g.*, in stably transfected aortic cells or isolated primary vascular smooth muscle cells) expressing the Deglycation Enzyme can be incubated in low glucose (*e.g.*, 5 mM) or conditions mimicking hyperglycemia (*e.g.*, high glucose concentrations, for example, 30 mM) for several days. Effects of prolonged hyperglycemia may be confirmed through pathways known to act via protein kinase C, AGE formation, and the production of sorbitol.

Whether overexpression of Deglycation Enzyme sensitizes the cell to cellular damage through increased free radical formation can be determined by measuring superoxide or hydrogen peroxide production by several methods, such as cytochrome c reduction or by commercially available fluorescent probes (*e.g.*, dihydrorhodamine123 (Molecular Probes). Effects of Deglycating Enzyme overexpression on cellular stress may also be analyzed by proteomic analysis of treated and non-treated cells using two-dimensional gel electrophoresis.

To determine the formation of early glycation products in the glycated proteins and low molecular weight sugar amine, fructosamine content, for example, from a biological sample, is measured by using the Fructosamine Test (Hoffmann-La Roche, Switzerland). This test relies on the ability of fructosamine to act as a reducing agent in alkaline solution. The AGE content of glycated proteins is determined by a noncompetitive and competitive enzyme-linked immunosorbent assay (ELISA) using polyclonal anti-AGE antibody. In addition, method such as HPLC and mass spectrometry are used to measure free low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs.

Other functional analyses of Deglycating Enzyme include those set forth above and elsewhere herein, including but not limited to identification of structural regions involved in: phosphorylation; binding of molecular oxygen and consequent generation of hydrogen peroxide, superoxide, or other reactive oxygen species; measurement of kinase activity; determination of nucleotide triphosphate binding; determination of binding to low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins; and binding

to transition-metal ions, such as those of copper or iron. Methods of performing these analyses are known to those of skill in the art and/or are described herein.

Analogs of Deglycating Enzyme

Deglycating Enzyme and analogs thereof may be generated, for example, by synthetic or recombinant means (*e.g.*, single or fusion polypeptides). Polypeptides, especially shorter polypeptides up to about 50 amino acids, are conveniently made by chemical synthesis. See, for example, Atherton and Sheppard, *Solid Phase Peptide Synthesis: A Practical Approach*, New York: IRL Press, 1989; Stewart and Young: *Solid-Phase Peptide Synthesis 2nd Ed.*, Rockford, Illinois: Pierce Chemical Co., 1984; and Jones, *The Chemical Synthesis of Peptides*, Oxford: Clarendon Press, 1994. For example, to be prepared synthetically, Deglycating Enzyme and/or portions thereof may be synthesized using any of the commercially available solid phase techniques such as the Merrifield solid phase synthesis method, where amino acids are sequentially added to a growing amino acid chain (see, for example, Merrifield, *J. Am. Soc.* 85:2146-2149 (1963); Marglin, A. and Merrifield, R.B. *Annu. Rev. Biochem.* 39:841-66 (1970); and Merrifield R.B. *JAMA* 210(7):1247-54 (1969)). Variations of the Merrifield solid phase synthesis, for example Fmoc, may also be used to chemically synthesize Deglycating Enzyme and Deglycating Enzyme analogs. Equipment for automated synthesis of peptides or polypeptides is also commercially available from suppliers such as Perkin Elmer/Applied Biosystems (Foster City, CA) and may be operated according to the manufacturer's instructions. Confirmation of the identity of the newly synthesized Deglycating Enzyme peptides and Deglycating Enzyme analogs may be achieved by amino acid analysis, mass spectroscopy, Edman degradation, or by assessing biological function (*e.g.*, degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs, deglycation of glycated substrates, *etc.*).

Analogs of Deglycating Enzymes and of encoding polynucleotides are also within the scope of the present invention. Such analogs include functional equivalents of Deglycating Enzyme and of the polynucleotides described above. Analogs of Deglycating Enzyme may be made by substituting amino acids which do not substantially alter the bioactivity of the Deglycating Enzyme analog. Selection of amino acids for substitution can depend on the size, structure, charge, and can be either an amino acid found in nature or synthetic amino acid. Generally, amino acids which have

a similar charge (*e.g.*, hydrophobic for hydrophobic) or similar size (*e.g.*, isoleucine for leucine) can be selected for substitution. One or more substitutions can be made in a stepwise fashion or concurrently. Variations in the residues can be included in the peptide. For example, it is possible to substitute amino acids in a sequence with interchangable amino acids using conventional techniques. As an example, groups of amino acids that may be interchanged in addition to those set forth elsewhere herein are:

Ala, Ser, Thr, Pro, and Gly;

Asn, Asp, Glu, and Gln;

His, Arg, and Lys;

Met, Glu, Ile, and Val; and

Phe, Tyr, and Trp

It is understood that many Deglycating Enzyme analogs can be achieved by substituting one or more amino acids. The Deglycating Enzyme analogs can be tested for biological function (*e.g.*, modification or degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins). The biological activity of a Deglycating Enzyme analog is at least about 25% of Deglycating Enzyme, preferably at least about 35%, preferably at least about 50%, preferably at least about 60%, preferably at least about 75%, preferably at least about 80%, preferably at least about 85%, preferably at least about 90%, and more preferably at least about 95%.

The invention also encompasses bioactive or functional fragments with Deglycating Enzyme bioactive functionality. Such bioactive or functional fragments may be obtained by deletion of one or more amino acid residues of full-length Deglycating Enzyme. Bioactive or functional fragments or portions of Deglycating Enzyme may be ascertained by stepwise deletions of amino acid residues, from the N-terminal end or the C-terminal end or from within the Deglycating Enzyme peptide. If an amino acid is deleted and the bioactivity of Deglycating Enzyme is not substantially reduced, then the amino acid may not comprise a portion of the bioactive or functional fragment. Further, polypeptides comprising a bioactive or functional fragment of Deglycating Enzyme or Deglycating Enzyme analog(s) are also encompassed in the invention.

Polynucleotides and polypeptides encoding Deglycation Enzyme

The invention also encompasses polynucleotides that code for Deglycating Enzyme or a bioactive or functional fragment of Deglycating Enzyme, or analog or derivative or variant. Polynucleotides which code for Deglycating Enzyme analogs and variants or bioactive or functional fragments of Deglycating Enzyme analogs and variants are also encompassed within the invention.

Deglycating Enzymes and related molecules may also be produced recombinantly by inserting a polynucleotide (usually DNA) sequence that encodes the polypeptide into a vector, for example, an expression vector and expressing the vector in an appropriate host. A polynucleotide encoding the desired polypeptide, whether in fused or mature form, and whether or not containing a signal sequence to permit secretion, may be ligated into expression vectors suitable for any convenient host. Any of a variety of expression vectors (either eukaryotic or prokaryotic) known to those of ordinary skill in the art may be employed. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a DNA molecule which encodes the recombinant peptides. Suitable host cells include prokaryotes, yeasts and eukaryotic cells. Examples of prokaryotic host cells are known in the art and include, for example, *E. coli* and *B. subtilis*. Examples of eukaryotic host cells are known in the art and include yeast, avian, insect, plant, and animal cells such as COS7, HeLa, CHO and other mammalian cells. Standard techniques for recombinant production are described for example, in Maniatis *et al.*, "Molecular Cloning - A Laboratory Manual," Cold Spring Harbour Laboratories, Cold Spring Harbour, New York (1989).

The polypeptide may then be isolated from lysed cells or from the culture medium, for example, and purified to the extent needed for its intended use. Purification or isolation of the polypeptides expressed in host systems can be accomplished by any method known in the art. For example, cDNA encoding a polypeptide intact or a fragment thereof can be operatively linked to a suitable promoter, inserted into an expression vector, and transfected into a suitable host cell. The host cell is then cultured under conditions that allow transcription and translation to occur, and the desired polypeptide is recovered. Other controlling transcription or translation segments, such as signal sequences that direct the polypeptide to a specific cell compartment (*e.g.*, for secretion), can also be used. In addition, other sequences which determine whether

Deglycating Enzyme is intracellular, secretory, soluble, or membrane-bound can be used depending on the desired outcome. For example, if a soluble Deglycating Enzyme or related peptide is desired, then the protein or peptide excludes the transmembrane sequence.

A fusion protein may also be constructed that facilitates purification. Examples of components for these fusion proteins include, but are not limited to myc, HA, FLAG, His-6, glutathione S-transferase, maltose binding protein or the Fc portion of immunoglobulin. These methods are known in the art. See, for example, Redd *et al. J. Biol. Chem.* 272:11193-11197 (1997).

If Deglycating Enzymes or related polypeptides are used for diagnostic purposes, the polypeptides are at least partially purified or isolated from other cellular constituents. Preferably, the polypeptides are at least about 50% pure. In this context, purity is calculated as a weight percent of the total protein content of the preparation. More preferably, the proteins are 50-75% pure. More highly purified polypeptides may also be obtained and are encompassed by the present invention. The polypeptides are preferably highly purified, at least about 80% pure, and free of pyrogens and other contaminants. Methods of protein purification are known in the art and are not described in detail herein.

Polypeptides which are homologous to Deglycating Enzyme but which, when bound to NEGs, for example, do not affect degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs, are also encompassed within the scope of the invention. Polypeptides may act as reversible (*e.g.*, competitive inhibitors, non-competitive inhibitors, or uncompetitive inhibitors) or irreversible inhibitors of Deglycating Enzyme binding to low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs. These polypeptides may be targeted to the active site or non-active site of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs, for example. Also encompassed within the scope of the invention are polynucleotides that encode the peptides, inhibitory polypeptides, vectors which comprise the polynucleotides which encode the peptides or inhibitory polypeptides, and host cells which comprise the vectors which comprise the polynucleotides that encode the peptides or inhibitory polypeptides.

Host cells comprising Deglycating Enzyme polynucleotides

Another aspect of this invention are host cells transformed or transfected with (*i.e.*, comprising) polynucleotides coding for Deglycating Enzyme or Deglycating Enzyme analogs, fragments, variants, homologues and derivatives, polynucleotides coding for bioactive or functional fragments of Deglycating Enzyme or bioactive or functional fragments of Deglycating Enzyme analogs, vectors comprising polynucleotides coding for bioactive or functional fragments of Deglycating Enzyme or bioactive or functional fragments of Deglycating Enzyme analogs, or other vectors as described above, and so on. Both prokaryotic and eukaryotic host cells may be used. Prokaryotic hosts include bacterial cells, for example *E. coli* and *B. subtilis*. Among eukaryotic hosts are yeast, insect, avian, plant and mammalian cells. One example of a mammalian host cell is NS0, obtainable from the European Collection of Cell Cultures (England). Transfection of NS0 cells with a plasmid, for example, which is driven by a cytomegalovirus (CMV) promoter, followed by amplification of this plasmid in using glutamine synthetase provides a useful system for protein production. Cockett *et al.* *BioTechnology* 8:662-667 (1990).

The host cells of this invention can be used, *inter alia*, as repositories of Deglycating Enzyme polynucleotides and/or vehicles for production of Deglycating Enzyme polynucleotides and related polypeptides. They may also be used as vehicles for *in vivo* delivery of various Deglycating Enzyme polypeptides.

Isolation of Deglycating Enzyme Polynucleotides

Deglycating Enzyme polynucleotides can be isolated from various sources including but not limited to host cells transformed (or transfected) with expression vector expressing Deglycating Enzyme, circulating Deglycating Enzyme *in vivo*, biological samples or fluids, tissue samples (*e.g.*, pancreas, lung, aorta, or liver), or from a cloning vector. Isolation of nucleotides from cells is routine to a skilled artisan and may be achieved using any number of commercially available nucleotide isolation kits, for example from Qiagen (Valencia, CA) or Promega (Madison, WI). Polynucleotides encoding Deglycating Enzyme may be in the form of DNA, RNA, DNA analogs, RNA analogs, or a hybrid of DNA-RNA. Deglycating Enzyme polynucleotides can also be single stranded or double stranded.

In one aspect, the Deglycating Enzyme of the invention comprises 309 amino acids. However, Deglycating Enzyme comprising more than 309 amino acids or less than 309 amino acids are within the scope of the invention. For example, in terms of Deglycating Enzyme itself, functional equivalents include all proteins that function as Deglycating Enzyme and have a minimum of 6 amino acids as disclosed in SEQ ID NO:1. In general, the functional equivalents have a minimum of 8 amino acids, more typically 10, 25, 30, 35, 40, 45, 65, 75, 95, 115, 145, 175, 195, 205, 215, 245, 275, 280, 285, 290, 295,300, 310, 302, 303, 304, 305, 306, 307, or 308 amino acids. In another aspect, the Glycating Enzyme comprises up to about 315 or more amino acids, and up to about 320 or more amino acids. In another aspect, the Deglycating Enzyme comprises a polypeptide according to SEQ ID NO:1 with one to five conservatively substituted amino acid residues. In another aspect, the Deglycating Enzyme comprises a polypeptide according to SEQ ID NO:1 with with an addition or deletion of one to ten amino acid residues, or an addition or deletion of one to five amino acid residues. In another aspect, the Deglycating Enzyme comprises a polypeptide which is generally at least 80% identical to SEQ ID NO:1, typically at least 85% identical to SEQ ID NO:1, more typically at least 90% identical to SEQ ID NO:1, and most typically least 95% identical to SEQ ID NO:1.

Preferably, these functional equivalents are immunologically cross-reactive. For example, these functional equivalents can include but are no limited to fragment of Deglycating Enzyme which can include Deglycating Enzyme active site(s), substitution, addition or deletion mutant of Deglycating Enzyme, fusion of Deglycating Enzyme, or a fragment or a mutant with other amino acids.

The six amino acids forming the smallest fragment can be from any part of the sequence, provided they are consecutive in that sequence and fulfil the functional requirement. The bioactive peptide can include any one of the hexapeptides, or include any heptapeptide, octapeptide, nonapeptide, decapeptide, or larger peptide from the sequence.

Equivalent polynucleotides include nucleic acid sequences that encode proteins equivalent to Deglycating Enzyme as defined above. Equivalent polynucleotides also include nucleic acid sequences that, due to the degeneracy of the nucleic acid code,

differ from native polynucleotides in ways that do not effect the corresponding amino acid sequences.

A prediction of whether a particular polynucleotide or polypeptide is equivalent to those given above can be based upon homology. Polynucleotide or polypeptide sequences may be aligned, and percentage of identical nucleotides in a specified region may be determined against another sequence, using computer algorithms that are publicly available. Two exemplary algorithms for aligning and identifying the similarity of polynucleotide sequences are the BLASTN and FASTA algorithms. The similarity of polypeptide sequences may be examined using the BLASTP algorithm. Both the BLASTN and BLASTP software are available on the NCBI anonymous FTP server (ftp://ncbi.nlm.nih.gov) under /blast/executables/. The use of the BLAST family of algorithms, including BLASTN and BLASTP, is described at NCBI's website at URL http://www.ncbi.nlm.nih.gov/BLAST/newblast.html and in the publication of Altschul, Stephen F, *et al* (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res.* 25:3389-3402. The computer algorithm FASTA is available on the Internet at the ftp site ftp://ftp.virginia.edu.pub/fasta/. The use of the FASTA algorithm is described in the W R Pearson and D.J. Lipman, "Improved Tools for Biological Sequence Analysis," *Proc. Natl. Acad. Sci. USA* 85:2444-2448 (1988) and W.R. Pearson, "Rapid and Sensitive Sequence Comparison with FASTP and FASTA," *Methods in Enzymology* 183:63-98 (1990).

Analogs according to the invention also include the homologues of Deglycating Enzyme from species other than human. Such homologues can be readily identified using, for example, nucleic acid probes based upon the conserved regions of the polynucleotides which encode human Deglycating Enzyme. Example 9 and Figure 4 illustrate the use of human Deglycating Enzyme sequence for alignment with sequences from other animals.

Deglycating Enzyme or its analogs, for example, and other related polypeptides, can also be present in various degrees of purity. Preferably, the Deglycating Enzyme/analog or related polypeptide component makes up at least 50% by weight of the preparation, more preferably at least 80% by weight, still more preferably at least 90% by weight, still more preferably at least 95% by weight and yet more preferably at least 99% by weight. It is however generally preferred that, for pharmaceutical

application, the Deglycating Enzyme or analog or related polypeptide be present in a pure or substantially pure form.

Methods of Using Deglycating Enzymes

As described above, the present invention provides an isolated substantially pure composition of Deglycating Enzymes and related polypeptides such as those described or referenced herein. This enzyme plays a physiological role in the modification or degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing proteins. It may also play a role as described herein in the subsequent generation of reactive oxygen species which can cause tissue toxicity and damage.

Accordingly, Deglycating Enzymes or related molecules may be used for identifying and/or screening for agents which modulate the activity of Deglycating Enzyme as described and/or claimed herein. It is understood that modulation of activity encompasses reduction, antagonism, or inhibition of one or more Deglycating Enzyme activitities as well as activation and enhancement. Various methods may be used to identify agents which modulate the activity of Deglycating Enzymes, for example. One method is to obtain *ex vivo* biological samples from individuals (*e.g.*, non-diabetic and diabetic) and determine the amount of free low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing molecules, or reactive oxygen species in each biological sample prior to exposure to a candidate agent. Next, a pre-determined amount of candidate agent is admixed with the biological samples and the amount of free low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing molecules, or reactive oxygen species in the sample is determined using the methods disclosed herein. Comparison between the amount of free low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing molecules, or reactive oxygen species in the biological samples can be made to determine if the candidate agent has any effect on the activity of Deglycating Enzyme. The amount of candidate agent can be modified in stepwise increment or decrements to obtain results over a range of concentrations.

In yet another aspect, Deglycating Enzyme and its analogs can also be used in methods for screening for and/or identifying agents which antagonize the activity of Deglycating Enzyme. Generally, an agent is selected for testing, then a pre-determined amount of the agent is admixed with Deglycating Enzyme, then the amount of free low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs, , or reactive oxygen species is measured or monitored to select the agent which results in lowered reactive oxygen species or reduced degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs.

Since the moldification degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing molecules may result in the generation of reactive oxygen species, an agent which inhibits or antagonizes the degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs may be selected for inclusion in pharmaceutical compositions, medicaments, and in treatment or prophylactic regimen. In the context of the present invention, agents include but are not limited to a biological or chemical compound such as a simple or complex organic or inorganic molecule, a peptide, a protein, an oligonucleotide, an antibody, an antibody derivative, or antibody fragment.

Deglycating Enzyme and its analogs can also be targets for drug screening purposes. Accordingly, methods of screening agents which are drug candidates are described herein. In one aspect, drug candidates are determined by identification of agents that promote or antagonize modification or degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs or AGEs or glycoproteins or other sugar-bearing molecules.

Without being bound by theory or mechanism, antagonism can occur by the agent interacting with and/or bind to a Deglycating Enzyme and competing for binding to low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs.

In another aspect, drug candidates are determined by identification of agents which interact with and/or bind to a protein or receptor associated with Deglycating Enzyme or its analogs such that antagonism of degradation of low molecular weight

amines and amino acids including fructosamines and other sugar amines or NEGs, for example, occurs.

One method which can be used for drug screening involves using mammalian cells which expresses Deglycating Enzyme or its analogs and contacting these cells with one or more drug candidate(s), and monitoring the effect of the drug candidate(s). Effects which can be monitored or measured include but are not limited to the amount of phosphorylation or degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs; amount of free low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs; consumption of molecular oxygen; amount of reactive oxygen species generated; measurement of cellular stress, (for example, by proteomic analysis of activity or content of Cu/Mn superoxide dismutase).

Accordingly, a method of drug screening involves mammalian cells expressing Deglycating Enzyme or its analogs exposed to one or a plurality of drug candidates, then determining those drug candidates which bind to Deglycating Enzyme or its analogs expressed in the mammalian cell, and thereby identifying drugs which interact with and/or bind to Deglycating Enzyme and its analogs. In another aspect, the drugs can bind to a protein associated with Deglycating Enzyme or its analogs (*e.g.*, transporter or receptor) and exert its effects.

Various methods of detection may be employed. The drugs may be labeled with a detectable marker (*e.g.*, radiolabel or a non-isotopic label such as biotin or fluorescent marker). Drug candidates can be identified by choosing agents which bind with affinity, preferably high affinity, to the Deglycating Enzyme and its analogs expressed in the cell, using techniques well known in the art. Drug candidates can also be screened for selectivity by identifying agents which bind to Deglycating Enzyme and its analogs but do not bind to any other receptors or receptor sites.

Deglycating Enzyme can also be used to determine the rate or amount of modulatory activity that a candidate agent has on Deglycating Enzyme activity. Such determinations can be made using *ex vivo* biological samples, in vitro samples (*e.g.*, recombinant proteins), synthetic samples, and the like. These determinations can be achieved by measuring or monitoring several factors before and after exposure to the candidate agent. The factors which can be measured or monitored include but are not

limited to: the rate and amount of degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs ; amount of free low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs; amount of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs; amount and rate of reactive oxygen species generated; rate of conversion from glycated to unglycated low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs. Methods of determining these factors are disclosed herein.

This invention also provides a pharmaceutical composition comprising a drug identified by the method described above and a pharmaceutically acceptable carrier. As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. Excipients as well as formulations for parenteral and nonparenteral drug delivery are set forth in *Remington's Pharmaceutical Sciences,* 19th Ed. Mack Publishing (1995). Once the candidate drug has been shown to be adequately bio-available following a particular route of administration, for example orally or by injection and has been shown to be non-toxic and therapeutically effective in appropriate disease models, the drug may be administered to patients by that route of administration determined to make the drug bio-available, in an appropriate solid or solution formulation, to gain the desired therapeutic benefit.

Disease models which may be used to assess efficacy of drug candidates include diabetic animal model (*e.g.*, streptozocin-induced diabetes in rats or mice, human amylin transgenic mice)

The invention also provides therapeutic treatment for an individual who has complication associated with diabetes. Non-limiting examples of complications associated with diabetes include: macrovascular and microvascular damage, cardiomyopathy, renal dysfunction, renal failure, blindness, gangene-related amputations, coronary heart disease, and ischemic brain damage. Accordingly, the invention also provides therapeutic treatment for an individual who is need of such therapy. Candidate individuals include diabetics, individuals with disease states associated with abnormally high or low Deglycating Enzyme activity, and individuals

with a condition which results in or involves undesired amount (high or low) of phosphorylation or degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs, for example. Therapeutic treatment can entail administration of an effective amount of an agent which has one or more Deglycating Enzyme activities.

Therapeutic treatment can also entail administration of an effective amount of an agent which antagonizes one more more Deglycating Enzyme activities. It is understood that administration of an agent which antagonizes Deglycating Enzyme activity will, in most cases, modulate low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs. Treatment with an effective amount of an agent which antagonizes Deglycating Enzyme activity is intended to palliate damage resulting from Deglycating Enzyme activity and reducing the severity of a symptom associated with damage resulting from Deglycating Enzyme activity in an individual. Treatment with an effective amount of an agent which antagonizes Deglycating Enzyme activity can also be administered in conjunction with other agents, for example, anti-diabetic or hypoglycemic drugs (*e.g.*, insulin, sulfonylureas, glitazones, biguanidines (*e.g.*, metformin), or pramlintide). Other classes of agents with which anti-Deglycating Enzyme agents may be used in conjunction with include but are not limited to anti-hypertensives, lipid lowering agents, anti-arrhythmics, calcium channel blockers, and anti-heart failure medication. Agents which antagonizes Deglycating Enzyme or an analog thereof and methods of obtaining these agents are described in greater detail below.

Further, the present invention provides methods for identifying for proteins (*e.g.*, receptors, agonists, antagonists, transcription factors, *etc.*) which interact with Deglycating Enzyme. Proteins which interact with Deglycating Enzyme can be determined by admixing a pre-determined amount of Deglycating Enzyme with a biological sample and detecting the interaction of Deglycating Enzyme with one or more components of the biological sample by standard methods known in the art (*e.g.*, denaturing and non-denaturing protein gels, Western blots, *etc.*). Additionally, tissue or cellular distribution may be ascertained by similar means. Similarly, nucleic acids which interact with Deglycating Enzyme may be determined in the same manner.

Deglycating Enzyme sequences may also be used as probes for finding other homologous proteins or sequences in other species other than human. This is exemplified in Example 9. Once homologous Deglycating Enzyme is found in other animals, for example mouse, then genetic tests can be conducted to determine the role of the Deglycating Enzyme gene. Mutagenesis of the Deglycating Enzyme gene or knockout animals (*e.g.*, mice) can be made to obtain more information about the role of the Deglycating Enzyme gene. Additionally, the probes may be used to find transcriptional or translational variants in the human genome (as shown in Example 10) or the genome of other animals or organisms (*e.g.*, bacteria, yeast, *etc.*)

Agents which antagonize Deglycating Enzyme activity

Agents which antagonize Deglycating Enzyme activity can be identified and screen using the methods described supra. Accordingly, the invention encompasses a variety of agents which can antagonize Deglycating Enzyme activity. Non-limiting examples of such agent include antibodies and polypeptides which are homologous to Deglycating Enzyme.

Antibodies can encompass monoclonal antibodies, polyclonal antibodies, antibody fragments (*e.g.*, Fab, Fab', $F(ab')_2$, Fv, Fc, *etc.*), chimeric antibodies, single chain (ScFv), mutants thereof, fusion proteins comprising an antibody portion, humanized antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site of the required specificity. The antibodies may be murine, rat, human, or any other origin. For purposes of this invention, the antibody is cross-reactive with Deglycating Enzyme in a manner that inhibits the degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs by Deglycating Enzyme. In one aspect, the antibody is a human or humanized monoclonal antibody which recognizes one or more epitopes on Deglycating Enzyme. In another aspect, the antibody is a mouse or rat monoclonal antibody which recognizes one or more epitopes on Deglycating Enzyme. The epitope(s) can be continuous or discontinuous. Examples of epitopes to which an antibody may be directed include the active site of Deglycating Enzyme. In another aspect, antibodies (*e.g.*, human, humanized, mouse, chimeric) which can inhibit Deglycating Enzyme interaction with NEGs may be made by using substantially pure Deglycating Enzyme as an immunogens or alternatively, cells which express

Deglycating Enzyme may be used as immunogens. Cells (*e.g.*, Raji cells) which can be obtained from ATCC (accession #CCL-86), may be transfected with a vector which encodes Deglycating Enzyme and transfected (stable or transient) may be used as immunogens. Expression levels of Deglycating Enzyme may be ascertained at regular intervals of time by running protein gels. Another example of an immunogen which can be used is a soluble Deglycating Enzyme fusion protein. Deglycating Enzyme or portions thereof may be fused with heavy chain IgG, for example, as disclosed in WO 91/16437. Raji cells expressing Deglycating Enzyme or Deglycating Enzyme fusion protein may be used alone or in combination with each other as immunogens.

The route and schedule of immunization of the host animal are generally in keeping with established and conventional techniques for antibody stimulation and production. It is contemplated that any mammalian subject including humans or antibody producing cells therefrom can be manipulated to serve as the basis for production of mammalian, including human, hybridoma cell lines. Typically, the host animal is inoculated intraperitoneally with an amount of immunogen sufficient to generate an immunogenic response and then boosted with similar amounts of the immunogen. Lymphoid cells, preferably spleen lymphoid cells from the host, are collected a few days after the final boost and a cell suspension is prepared therefrom for use in the fusion.

Hybridomas can be prepared from the lymphocytes and immortalized myeloma cells using the general somatic cell hybridization technique of Kohler, B. and Milstein, C. *Nature* 256:495-497 (1975) or as modified by Buck, D. W., *et al. In Vitro,* 18:377-381 (1982). Available myeloma lines, including but not limited to X63-Ag8.653 and those from the Salk Institute, Cell Distribution Center, San Diego, Calif., USA, may be used in the hybridization. Generally, the technique involves fusing myeloma cells and lymphoid cells using a fusogen such as polyethylene glycol, or by electrical means well known to those skilled in the art. After the fusion, the cells are separated from the fusion medium and grown in a selective growth medium, such as hypoxanthine-aminopterin-thymidine (HAT) medium, to eliminate unhybridized parent cells. Any of the media described herein, supplemented with or without serum, can be used for culturing hybridomas that secrete monoclonal antibodies. As another alternative to the cell fusion technique, EBV immortalized B cells may be used to produce the anti-Deglycating

Enzyme monoclonal antibodies of the subject invention. The hybridomas are expanded and subcloned, if desired, and supernatants are assayed for anti-immunogen activity by conventional immunoassay procedures (*e.g.*, radioimmunoassay, enzyme immunoassay, or fluorescence immunoassay).

Hybridomas that may be used as source of antibodies encompass all derivatives, progeny cells of the parent hybridomas that produce monoclonal antibodies specific for antigens representative of the type of cells used for immunization.

Hybridomas that produce such antibodies may be grown *in vitro* or *in vivo* using known procedures. The monoclonal antibodies may be isolated from the culture media or body fluids, by conventional immunoglobulin purification procedures such as ammonium sulfate precipitation, gel electrophoresis, dialysis, chromatography, and ultrafiltration, if desired. Undesired activity if present, can be removed, for example, by running the preparation over adsorbents made of the immunogen attached to a solid phase and eluting or releasing the desired antibodies off the immunogen. Immunization of a host animal with cells expressing Deglycating Enzyme or Deglycating Enzyme fusion proteins can yield a population of antibodies (*e.g.*, monoclonal antibodies).

In an alternative, antibodies may be made recombinantly and expressed using any method known in the art. Antibodies may be made recombinantly by first isolating the antibodies made from host animals, obtaining the gene sequence, and using the gene sequence to express the antibody recombinantly in host cells (*e.g.*, CHO cells). Another method which may be employed is to express the antibody sequence in plants (*e.g.*, tobacco) or transgenic milk. Methods for expressing antibodies recombinantly in plants or milk have been disclosed. See, for example, Peeters, *et al.* (2001) *Vaccine* 19:2756; Lonberg, N. and D. Huszar *Int.Rev.Immunol* 13:65 (1995); and Pollock, *et al. J Immunol Methods* 231:147 (1999). Methods for making derivatives of antibodies, *e.g.*, humanized, single chain, *etc.* are known in the art.

If desired, the anti-Deglycating Enzyme antibody (monoclonal or polyclonal) of interest may be sequenced and the polynucleotide sequence may then be cloned into a vector for expression or propagation. The sequence encoding the antibody of interest may be maintained in vector in a host cell and the host cell can then be expanded and frozen for future use. In an alternative, the polynucleotide sequence may be used for genetic manipulation to "humanize" the antibody. For example, the constant region may

be engineered to more resemble human constant regions to avoid immune response if the antibody is used in clinical trials and treatments in humans. It may be desirable to genetically manipulate the antibody sequence to obtain greater affinity to low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs.

There are four general steps to humanize a monoclonal antibody. These are: determining the nucleotide and predicted amino acid sequence of the starting antibody light and heavy variable domains, designing the humanized antibody, *i.e.*, deciding which antibody framework region to use during the humanizing process, the actual humanizing methodologies/techniques and the transfection and expression of the humanized antibody. For example, the constant region may be engineered to more resemble human constant regions to avoid immune response if the antibody is used in clinical trials and treatments in humans. See, for example, U.S. Patent Nos. 5,997,867 and 5,866,692. It will be apparent to one of skill in the art that one more polynucleotide changes can be made to Deglycating Enzyme and still maintain its binding ability to low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs.

The antibodies made either by immunization of a host animal or recombinantly exhibit one or more of the following characteristics, for example: (a) binds to Deglycating Enzyme; (b) binds to one or more epitopes of Deglycating Enzyme which interact with low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs; (c) binds to Deglycating Enzyme to antagonize Deglycating Enzyme activity; (d) binds to Deglycating Enzyme to antagonize Deglycating Enzyme activity and reduce the amount of reactive oxygen species generated; and (e) binds to Deglycating Enzyme to antagonize Deglycating Enzyme activity and reduce the amount of reactive oxygen species generated and the sequelae associated with reactive oxygen species.

Immunoassays and flow cytometry sorting techniques such as fluorescence activated cell sorting (FACS) can also be employed to isolate antibodies that are specific for Deglycating Enzyme. For example, ELISA plates coated with fragments of Deglycating Enzyme may be employed to determine which antibodies are specific for Deglycating Enzyme. Flow cytometry may be used to assess how well the antibody(-

ies) bind to Deglycating Enzyme-expressing cells. Flow cytometry may be used to detect how well the anti-Deglycating Enzyme antibody inhibits Deglycating Enzyme interaction with low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs.

The antibodies can be bound to many different carriers. Carriers can be active and/or inert. Examples of well-known carriers include polypropylene, polystyrene, polyethylene, dextran, nylon, amylases, glass, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding antibodies, or will be able to ascertain such, using routine experimentation.

The antibodies can also be conjugated to a detectable agent. The complex is useful to detect the antigens to which the antibody specifically binds in a sample, using standard immunochemical techniques such as flow cytometry or immunohistochemistry as described by Harlow and Lane (1988) *supra*. Detectable markers can also be used to ascertain binding specificity for a type of cell (*e.g.*, islet cell) by using the detectable marker with another marker which is definitive for that cells (*e.g.*, anti-insulin antibody) and analyzing the staining patterns by FACS. There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include radioisotopes, enzymes, colloidal metals, fluorescent compounds (*e.g.*, FITC, PE, PECy5, APC, *etc.*), bioluminescent compounds, and chemiluminescent compounds. Those of ordinary skill in the art will know of other suitable labels for binding to the antibody, or will be able to ascertain such, using routine experimentation. Furthermore, the binding of these labels to the antibody of the invention can be done using standard techniques common to those of ordinary skill in the art.

Administration of agents

Various formulations of agents such as polypeptides, antibodies, or fragments thereof may be used for administration. In some aspects, agent(s) may be administered neat.

In other aspects, the agents comprise anti-Deglycating Enzyme antibodies or fragments thereof and a pharmaceutically acceptable excipient, and may be in various formulations.

Pharmaceutically acceptable excipients are known in the art, and are relatively inert substances that facilitate administration of a pharmacologically effective substance. For example, an excipient can give form or consistency, or act as a diluent. Suitable excipients include but are not limited to stabilizing agents, wetting and emulsifying agents, salts for varying osmolarity, encapsulating agents, buffers, and skin penetration enhancers. Excipients as well as formulations for parenteral and nonparenteral drug delivery are set forth in *Remington's Pharmaceutical Sciences,* 19th Ed. Mack Publishing (1995).

Generally, these agents are formulated for administration by injection (*e.g.*, intraperitoneally, intravenously, subcutaneously, intramuscularly, *etc.*). Accordingly, these agents are preferably combined with pharmaceutically acceptable vehicles such as saline, Ringer's solution, dextrose solution, and the like. The particular dosage regimen, (*i.e.*, dose, timing and repetition), will depend on the particular individual and that individual's medical history. Empirical considerations, such as the half life, generally will contribute to determination of the dosage. Frequency of administration may be determined and adjusted over the course of therapy, and is based on maintaining reduction of Deglycating Enzyme activity and suppression/amelioration/delay of one or more symptoms associated with abnormal Deglycating Enzyme activity. Alternatively, sustained continuous release formulations of the agents may be appropriate. Various formulations and devices for achieving sustained release are known in the art.

In an alternative, dosing regime may be adjusted accordingly for an individual who has a family history of diabetes to delay development of the sequelae associated with diabetes. It will be apparent to one of skill in the art that the dosage will vary depending on the individual, the stage of diabetes, and type of diabetes within the individual. In such cases, consideration is made to balance side effects resulting from possible toxicity with an effective dosage .

In one aspect, dosages for agents may be determined empirically in individuals who have been given one or more administration(s) of an agent which antagonizes Deglycating Enzyme activity to treat diabetes. Individuals are given incremental

dosages of an agent which antagonizes Deglycating Enzyme activity, *e.g.*, anti-Deglycating Enzyme antibody. In one aspect, the antibody bind to an active site in Deglycating Enzyme and inhibits or antagonizes its activity. In another aspect, the agent (*e.g.*, antibody) binds to another site in Deglycating Enzyme and inhibits or antagonizes its activity by another mechanism. Without being bound by theory, other mechanisms which could affect the activity of Deglycating Enzyme include but are not limited to conformational disruption, reversible (*e.g.*, competitive inhibitors, non-competitive inhibitors, or uncompetitive inhibitors) or irreversible inhibition, and targeting the Deglycating Enzyme for an alternate pathway (*e.g.*, destruction by proteasomes).

Other formulations include suitable delivery forms known in the art including, but not limited to, carriers such as liposomes. Mahato *et al.* (1997) *Pharm. Res.* 14:853-859. Liposomal preparations include, but are not limited to, cytofectins, multilamellar vesicles and unilamellar vesicles.

In some aspects, more than one agent, such as an antibody, may be present. The agents can be the same or different from each other. Such agents may contain at least one, at least two, at least three, at least four, at least five different antibodies. Anti-Deglycating Enzyme antibody can be admixed with one or more antibodies also reactive against Deglycating Enzyme but with differing epitopes. In another aspect, the agent is a polypeptide which promotes or antagonizes a Deglycating Enzyme activity. The polypeptide may be admixed with another agent, for example, an agent with SOD activity to achieve a more beneficial clinical effect.

Methods of assessing efficacy of treatment

Assessment of treatment efficacy can be performed on several different levels. Assessment may be made by monitoring clinical signs (*e.g.*, symptoms associated with undesired or abnormal Deglycating Enzyme activity), cellular or tissue responses (*e.g.*, apoptosis and/or necrosis), or molecular changes within one or more cells (*e.g.*, phosphorylation). In one aspect, a baseline level of Deglycating Enzyme activity for an individual may be taken at one time or over a period of time such that the baseline level of Deglycating Enzyme activity may be used to determine if that individual develops abnormal Deglycating Enzyme activity, for example, when he/she develops diabetes or when the diabetes becomes more severe.

Detection and measurement of efficacy in treatment of diabetes or a disease state associated with undesired or abnormal Deglycating Enzyme activity can be achieved by monitoring factors including but not limited to amount of free low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs, amount of reactive oxygen species generated, amount of glycated protein, rate of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs degradation, rate of reactive oxygen species generated, and rate of glycated protein conversion to unglycated protein.

The following examples are provided to illustrate, but not limit, the invention.

EXAMPLES

Example 1 Cloning of Deglycating Enzyme gene

The Deglycating Enzyme gene was cloned through PCR amplification of total RNA isolated from SSH-7 lung carcinoma cells. The materials used were: Large cell variant of small cell lung carcinoma (ATCC Catalog No. CRL-2195); Trizol reagent (Roche Molecular Biochemicals Catalog No. 15596), Isopropanol (Sigma Catalog No. 1-9516); chloroform, 75% ethanol, 3'-RACE System (Invitrogen) Catalog No. 18373-027), sense primer: 5'-ATGGAGGAGCTGCTGAGG-3' (SEQ ID NO:3), antisense primer: TCACTTGACCAGATTCCTCATGATGTT (SEQ ID NO:4) (oligonucleotides were obtained commercially (Sigma)); Taq DNA polymerase (Roche Molecular Biochemicals Catalog No. 1146-165), pGEM-T Easy Vector cloning system (Promega Catalog No. A1360), DNA ligase (Roche); Normal Human Northern Blot I comprising a pre-made Northern blot filter of total RNA from several human tissues (Invitrogen Catalog No. D1100-01), ProbeQuant G-50 microColumn (Pharmacia Catalog No. 27-5335-01), ^{32}P radioactivity (Amersham Radiochemicals), QlAquick gel extraction kit (QIAGen Catalog No. 28704), High pure plasmid isolation Kit (Roche Catalog No.1 754 777), LB/Amp medium, LB/Amp/Xgal/IPTG plates, Type-II-A Agarose (Sigma Chemicals Catalog No. A-9918).

RNA was isolated from approximately 10^7 cells grown in suspension in RPMI medium through the Trizol method (Roche). Approximately 1 μg of total RNA was reverse transcribed using the 3'-RACE system according to manufacturers protocols. PCR amplification was then performed under the following conditions: 200 ☒M dNTP,

0.4 ☒M for both sense and antisense primers, ~100 ng cDNA, 2 U AmpliTaq, 1.5 mM $MgCl_2$. Fifty rounds of PCR amplification were performed as follows: 15 seconds at 94 °C, 30 seconds at 70 °C, 2 minutes at 72°C.

Purification of the resulting PCR products on a 1% agarose gel revealed the presence of an approximately 913 bp fragment (Fig. 1). This fragment was excised and purified using a QIAquick gel extraction kit, and ligated into pGEM vector according to manufacturers instruction. The ligated mixture was then used to transform bacterial DH5α-F'cells. Insert-containing colonies were selected using blue/white selection. Colonies were grown and plasmids isolated according to standard protocols. The identity of the enzyme was confirmed by dideoxy polynucleotide sequence analysis using base-specific fluorescent markers.

Example 2 Northern Analysis of total RNA from various human tissues

A 556 bp DNA fragment corresponding to the cloned Deglycating Enzyme gene was gel purified from a PstI restriction digest of the insert-containing pGEM plasmid using a QIAquick gel extraction kit (Fig 2).

Approximately 25ng of the purified DNA fragment was then ^{32}P radiolabelled using random primers according to standard protocols. The radiolabelled probe was then hybridized to a total RNA isolated from various human tissues (Clontech). Membrane was incubated in Church & Gilbert hybridization buffer for 1 hour at 65 °C prior to hybridization. ^{32}P radiolabel was then added (after heat denaturation) and hybridized overnight at 42 °C. The membrane was then washed twice in 2xSSC, 0.5% SDS for 30 minutes, then washed twice at high stringency (1 x SSC, 0.5% SDS) for 30 minutes at 65 °C. The blot was then visualized using a phosphorimager. The resulting blot revealed a radiolabelled species at approximately 1.8 kbp, showing that the gene was expressed in all the tissues probed (Fig. 3).

Example 3 Structure of Deglycation Enzyme

The gene encoding Deglycation Enzyme is expressed in both bacterial (*e.g.*, *E. coli*) and eukaryotic (*e.g.*, *Pichia methanolica*) cellular systems using appropriate expression vectors that are available commercially (*e.g.*, PET-17 and pMet). The Deglycation Enzyme gene product is isolated and purified from cellular lysates and purified according to standard techniques in protein chemistry. In addition, the

Deglycation Enzyme gene is modified using mutagenesis to include an epitope (*e.g.*, polyhistidine tag) on either the N-terminus or C-terminus. Deglycation Enzyme gene is expressed as a fusion protein in an appropriate vector construct (*e.g.*, as a fusion with Glutathione-S-transferase (GST) or maltose-binding protein). The resulting constructs are also expressed in the bacterial and eukaryotic systems. The epitope is used to facilitate purification of the gene products (*e.g.*, glutathione beads or column chelation of the histidine-tag epitope). Once purified, the recombinant protein is crystallized according to standard protocols (*e.g.*, hanging drop technique). Crystals are then subjected to X-ray diffraction and subsequent analysis to determine the three dimensional structure of the protein. This information is used in combination with other characterizations to identify the active site of Deglycation Enzyme, determine the other functions of Deglycation Enzyme , and serve as a basis for structure-based drug design.

Other expected functional analysis includes but is not limited to identification of structural regions involved in: binding of molecular oxygen and consequent generation of hydrogen peroxide or superoxide or other reactive oxygen species; kinase activity; nucleotide triphosphate binding; binding to low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs; binding to transition-metal ions, such as those of copper or iron. These regions are identified through co-crystallization of Deglycating Enzyme with nucleotide triphosphates, metal cations, or low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs, and solving the composite structure. Conventionally, it is normal for a critical active site residue to be mutated so that the enzyme is inactive to prevent catalysis.

Example 4 Expression of Deglycating Enzyme

Deglycating enzyme was expressed in SF-9 insect cells using a baculovirus expression system. The gene encoding F3KLP was cloned into the pFastbac Htb vector through NcoI and Eco RI restriction sites. The resulting construct was then used to create a recombinant baculovirus for subsequent expression of the recombinant protein in SF-9 insect cells. The construct encoded Deglycating Enzyme with a N-terminal six amino acid histidine tag to facilitate purification. The expressed protein with the histidine tag was termed F3KLPNHis.

For expression of Deglycating Enzyme, two 200 ml cultures of SF-9 cells were grown up to a density of 2×10^6 cells/ml in SF-900II SFM media, supplemented with penicillin and streptomycin. Cultures were infected with recombinant baculovirus at a dilution of 1:1500. Cultures were then incubated for 72 hours before harvesting the cells by centrifugation at 1000×g for 15 min at 4°C in a Sorvall SLA-3000 rotor. Pelleted cells were resuspended in 8ml of PBS containing protease inhibitors, and transferred to a 50ml Falcon Tube. Cells were then spun at 2000rpm in a Sorvall RTH-750 rotor for 10 min at 4°C, and the supernatant removed. Pelleted cells were suspended in an equal volume of phosphate-buffered saline (PBS). The resulting cell paste was then frozen at -80°C for storage.

Example 5 Purification of Deglycating Enzyme

The recombinant protein was purified to electrophoretic homogeneity using Cobalt affinity chromatography followed by anion exchange chromatography. The protocol relies on an initial cobalt affinity purification step, which is highly specific for recombinant proteins containing histidine tags.

Protease inhibitors (Roche, 1 tablet per 50ml) were added to 100ml of lysis buffer (50mM Tris, pH 8.0). Approximately 1.5 ml of Talon cobalt resin (Clontech 8901-2)was used to pack a 10 ml column. The packed column was washed with 10ml of Milli-Q water, and equilibrated with 10 column volumes of lysis buffer, followed by 2 column volumes of lysis buffer containing protease inhibitors.

Cell paste, prepared as described previously was weighed, and 25ml of lysis buffer added for every 6ml of cell paste. Cells were resuspended, and lysed by a single pass through a Constant Cell Disruption Systems cell disrupter. Cell debris was removed by centrifugation at 12,000×g for 10 min at 4°C in a Sorvall SS-34 rotor. The cleared cell lysate was applied to the equilibrated cobalt column. The flow through was collected and reapplied to the column a further two times. 10 column volumes of lysis buffer containing protease inhibitor tablets was used to wash the column, followed by 10 column volumes of lysis buffer without protease inhibitors. The flow through from each step was collected in 50 ml Falcon tubes, and stored at -80°C. Bound protein was eluted with 20 ml of Talon elution buffer (50mM Tris, pH 8.0, 80mM Imidazole, 50mM NaCl, 5% Glycerol) and collected as 1 ml fractions. 75ml of each fraction was removed for

evaluation of protein content by SDS-PAGE analysis and a bicinchonic acid (BCA) protein assay (Pierce). The N-terminal histidine-tagged protein eluted from the Cobalt affinity column was present predominantly in the first 8-9 fractions, with the majority of protein being present in fractions 2, 3, and 4. Aliquots were stored at -80°C for further purification.

Fractions shown to contain protein were desalted using a 1.5 ml HiTrap Desalting Column equilibrated with Q-Sepharose binding buffer (10 mM Tris, pH 8.0, 1 mM dithiothreitol). Desalted fractions were pooled and split into 2ml aliquots. There were no appreciable losses of protein noted during desalting. The recombinant protein was further purified by anion exchange chromatography using a BioCad Sprint chromatography system (PerSeptive). A 1.6ml ml column packed with Poros HQ-20 media was washed with 5 column volumes of Milli-Q water, and equilibrated with 10 column volumes of Q-Sepharose buffer A (10 mM Tris, pH 8.0, 1 mM dithiothreitol) . 4 ml of the pooled desalted fraction was applied to the equilibrated column through a 5 ml sample loop. The column was then washed with 3 column volumes of binding buffer before eluting the bound protein in a linear gradient of NaCl (0-500 mM) over 20 column volumes. A typical trace obtained during purification the Poros HQ-20 media is shown in Figure 6. The Deglycating Enzyme eluted as a single peak at the beginning of the salt gradient over 5 consecutive 2mL fractions.

Fractions corresponding to the major peak eluted from the Q-Sepharose column were analysed by running 15µl aliquots on 12% SDS-PAGE. Fractions showing a band of the correct molecular weight for the recombinant protein were pooled, and desalted using a HiTrap Desalting Column equilibrated with Buffer A. Centricon centrifugal concentrators were then used to concentrate the pooled desalted fractions to a final concentration of between 1 and 5 mg/ml as determined by the BioRad Rc/Dc protein assay. This final enzyme preparation was stored in aliquots at -80°C for later use.

Analysis of fractions in SDS-PAGE (Figure 7) showed that the recombinant protein was purified to electrophoretic homogeneity using the techniques described. The majority of cellular and viral proteins present in the crude cell lysate were removed during Cobalt affinity chromatography. The remaining contaminating proteins were effectively removed in the subsequent anion exchange step. Typically total yield of the purified protein was 2-3mg.

Example 6 Molecular Identification of Deglycating Enzyme by peptide mass fingerprinting

Peptide mass finger printing was used to confirm the identity of Deglycating Enzyme. Protein was digested with trypsin, and the size of the resulting peptides determined by mass spectrometry. Purified deglycating protein (2µg) was run on 12% SDS-PAGE, and visualized by Coomassie R250 staining. The band corresponding to the purified protein was excised and an in-gel tryptic digestion performed. The resulting peptides were analysed by mass spectrometry using a Voyager MALDI-TOF mass spectrometer (Applied Biosystems). Peptide masses of the resulting spectra were compared to theoretical masses generated using the web-based "PeptideCutter" program available from the Export Protein Analysis System proteomics server of the Swiss Institute of Bioinformatics. A number of good quality mass spectra were obtained from the tryptic digest of F3KLPNHis. From these spectra a number of clear peaks were identified. Experimentally derived masses of Deglycating Enzyme tryptic peptides showed good agreement with theoretical molecular masses. A typical mass spectum is shown in Figure 8. A list of the major peaks from this spectrum, and the corresponding theoretical peptide masses generated *in silico* are given in below.

Observed mass (Da)	**Theoretical Mass (Da)**	**Residues**	**Peptide sequence**
760.4655	760.4352	200-205	IPDLFR (SEQ ID NO: 7)
804.3158	804.4362	270-276	APGFEKR (SEQ ID NO: 8)
877.4998	877.4560	298-305	GSSLNIMR (SEQ ID NO: 9)
941.4429	941.3959	30-37	SYDTDQGR (SEQ ID NO: 10)
1302.7293	1302.6549	131-142	GGGQEERPFVAR (SEQ ID NO: 11)
1407.8459	1407.7590	103-115	LGAQLADLHLDNK (SEQ ID NO: 12)
1535.9426	1535.8540	103-116	LGAQLADLHLDNKK (SEQ ID NO: 13)
1540.8650	1540.7749	51-64	MFEGEMASLTAILK (SEQ ID NO: 14)
1696.9707	1696.8760	50-64	RMFEGEMASLTAILK SEQ ID NO: 15)
1944.1362	1944.0185	183-199	ESGDREALQLWSALQLK (SEQ ID NO: 16)
2014.0813	2013.9554	76-94	VLDAPGGGSVLVMEHMDMR (SEQID NO:17)

Example 7 Production of antibodies to Deglycation Enzyme

Purified Deglycation Enzyme is used as an immunogen to raise polyclonal antibodies using conventional techniques. In addition, fragments of the Deglycation Enzyme gene corresponding to surface epitopes are expressed as GST-fusion proteins and purified from bacterial systems using standard techniques. Constructs are made with the active site of Deglycation Enzyme. These constructs are used to raise polyclonal antibodies. Antibodies are affinity-purified, for example using protein-A Sepharose, using standard techniques.

Recombinant Deglycating Enzyme with a six amino acid N-terminal histidine tag was expressed and purified as described, and used to immunise a single male New Zealand White rabbit by subcutaneous injection. Two booster shots were given 6 weeks after the first immunisation. The rabbit was bled out days after the administration of the second booster, and serum collected by centrifugation. The presence of antibodies reactive towards histidine-tagged Deglycating Enzyme (F3KLPNHis) in the serum of the immunised rabbit was assessed by Western Blotting. Blots were performed using both purified histidine-tagged Deglycating Enzyme, and cell lysates from Cos-7 cell transiently transfected with a construct encoding Deglycating Enzyme with a C-terminal FLAG tag (F3KLPCFLAG). This was done in order to test whether the antibodies raised to the his-tagged protein were able to recognise the protein itself, and not the tag region. Lysates of Cos-7 cells were prepared by scraping cells from 100mm cell culture dishes containing either untransfected cells, transiently transfected cells into 10ml of PBS (10 mM Tris, pH 8.0) per dish. Cells were then pelleted by centrifugation at 3000 rpm for 5 min (4°C), and washed twice by resuspending in 10 ml of PBS. Following the final centrifugation, the cells were resuspended in 0.5 ml of 10mM Tris, pH 8.0, transferred to a 1.5 ml microfuge tube, and lysed by sonication. Cell debris was pelleted by centrifugation at 13,000 rpm for 5 min (4°C), and the cleared lysate transferred to a fresh microcentrifuge tube. 15µl of this preparation was loaded onto 12% SDS-PAGE for subsequent western blotting.

For lanes loaded with histidine-tagged Deglycating Enzyme, samples were prepared by adding 2μg of purified recombinant protein to 2μl of 4× reducing sample buffer, and a sufficient volume of 10mM Tris, pH 8.0 to give a final concentration of 1× for the sample buffer. Following the blotting and blocking steps, the membrane was transferred to a Hoeffer Deca-probe Incubation Manifold to allow different concentrations of anti-serum to be applied to different lanes of the blot. The anti serum was shown to detect purified F3KLPNHis down to a dilution of 1:10,000 as illustrated in Figure 9. In addition, the anti serum was also able to detect F3KLP expressed with a C-terminal FLAG tag in Cos-7 cells as a single band in a whole cell lysate. This result shows that the antibodies raised are highly specific to the protein itself, and not the tag portion of the recombinant protein.

Example 8 Regulation of Deglycation Enzyme

The regulation of Deglycation Enzyme is determined by using animal models of diabetes. In diabetic animal model (*e.g.*, streptozocin-induced diabetes in rats) and normal animal models, the expression pattern is analyzed using Northern and Western blotting, and *in situ* hybridization techniques. Endogenous expression and regulation in cell culture models in response to hyperglycemia conditions are also determined.

Example 9 Cell culture expression of Deglycation Enzyme

The Deglycation Enzyme gene is subcloned into an appropriate mammalian expression vector (*e.g.*, pcDNA 3.1) and the resulting constructs are transfected into cultured cells (*e.g.*, aortic endothelial cells Cos-7). Both transiently and stably transfected cells are generated using standard protocols known in the art. Stable transfection involves the use of a selectable marker, such as neomycin resistance. Cellular production of the gene and gene product are analyzed by Northern blotting, protein analysis by Western blotting, and direct sequence analysis. Cellular location is also analyzed to determine whether the Deglycation Enzyme gene product is secreted, intracellular, or attached to the cell membrane by analyzing fractionated portions of the cells in which Deglycation Enzyme is expressed with Northern or Western blots. Antibodies raised against the full length Deglycation Enzyme, a portion thereof, or from GST-fusions are used to examine cellular location using immunocytochemistry. Similar

experiments are performed on epitope-tagged constructs, such as through attachment of a flag or myc-epitope.

Example 10 Measurement of Kinase Activity

Fructosamine kinase activity was assayed using glycated lysozyme as a substrate since it is cationic, and is therefore easily separated from unreacted [γ33P]ATP. Enzyme preparations were incubated with glycated and non glycated lysozyme under the conditions outlined by Delpierre *et al* (Delpierre, G., *et al.*, *Diabetes* 49: 1627-1634 (2000)).

Reagents:

Starscint Liquid Scintilent (Packard Bioscience 6013248),

[γ33P]ATP (Amersham, BF1000-500μCi)

Hen Egg Lysozyme.

Fructosamine Assay Solution:

100mM Na2CO3 (BDH)

0.25% (w/v) Nitro Blue Tetrazolium (BioRad, 170-6532)

Made up in Milli-Q water and adjusted to pH 10.8

In vitro glycated lysozyme was produced by incubation of 60mg/ml lysozyme with 100mM D(+)glucose at 37°C under aseptic conditions for 20 days as outlined by Delpierre *et al* (Delpierre, G., *et al.*, *Diabetes* 49: 1627-1634 (2000)). 60mg/ml lysozyme and 100mM were dissolved in 50mM HEPES, pH 7.2. The solution was filtered through a 0.2μm filter directly into sterile 2ml cryovials before incubating for 20 days at 37°C. The presence of fructosamine glycated protein was then confirmed by diluting 100μl of the incubated solution into 1ml of fructosamine assay solution and looking for the presence of a blue colour change after 15 minutes incubation at 37°C. (This qualitative assay for detecting fructosamine in solution was based on the quantitative assay described by Johnson *et al* (Johnson, Metcalf *et al.* 1983)).

Kinase activity due to Deglycating Enzyme was detected by incubating 10μg of glycated lysozyme with 10μg of purified F3KLPNHis in the presence of [γ33P]ATP at 37°C for 30 min. No enzyme and no substrate controls were used as well as an additional control using non-glycated lysozyme instead of glycated lysozyme. The assay was performed as outlined:

1) A suitable volume of "kinase assay mastermix" was prepared according to the volumes shown in table 3.2, and dispensed into the required number of fresh 1.5ml microfuge tubes

2) The required amount of F3KLPNHis was thawed on ice

3) Tubes containing reaction mix were preincubated for 10 min at 37°C

4) 10μg of enzyme was added to each preincubated tube (in no enzyme controls, an equivalent volume of milli-Q water was added instead)

5) Tubes were incubated at 37°C for a further 30 min

6) After 30 min, the reaction was stopped by spotting the entire reaction mixture onto a 1.5cm2 piece of Whatman P81 paper

7) Spotted papers were allowed to air dry for 5 min before being placed in a beaker containing 50ml of ice cold milli-Q water

8) Papers were washed four times in 50mL of ice cold milli-Q water

9) After the last wash, papers were transferred to scintilation vials containing 2mL of scintillation liquid, and counted for activity.

Components of Fructosamine Kinase Assay "Master" Mixture for different reactions and controls was made up according to the volumes given below:

Component	Volume per reaction	**Final concentration**
250mM Tris, pH 7.8	5μl	25mM
50mM MgATP	0.1μl	100μM
0.5M EGTA	0.1μl	1mM
5mg/ml glycated lysozyme*	2μl	10μg/reaction
10mg/ml BSA	0.5μl	100μg/ml
[γ^{33}P]ATP	750,000dpm	750,000bpm/reaction
F3KLPNHis**	10μg	10μg/reaction
Milli-Q water	to 50μl	-

* Replaced with lysozyme incubated in absence of glucose under identical conditions as glycated lysozyme for non-glycated lysozyme control. Replaced with milli-Q water in no substrate controls.

** Replaced with milli-Q water for no enzyme controls.

Reactions containing glycated lysozyme, and F3KLPNH is showed a significantly higher rate of incorporation of ^{33}P than reactions containing non-glycated lysozyme, as illustrated in figure 10. This shows that Deglycating Enzyme recognises and phosphorylates glycated lysozyme but not native lysozyme, showing that Deglycating Enzyme is a fructosamine kinase.

Example 11 Measurement of Deglycation Activity

The function of the Deglycation Enzyme is measured through a series of assays using low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs as substrates. Examples of substrates are synthetic fructosamine and 1-deoxy-l-morpholinofructose (DMF). Activities of Deglycation Enzyme are measured using purified glycated substrates from either bacterial or eukaryotic (*e.g.*, yeast) sources, and on transfected cellular systems. For the latter, substrates are overlayed on the cultured cells and incubated under defined culture conditions. As substrate phosphorylation may be an intermediate step contributing to the deglycation process, enzyme-catalyzed phosphorylation of these substrates are measured following purification by anion-exchange chromatography or gel extrusion chromatography. Phosphorylation is measured by MALDI-TOF mass spectrometry, NMR, and in the case of glycated protein substrates, two-dimensional gel electrophoresis. Since fructose is a possible substrate for this enzyme, phosphorylation of fructose is also measured using MALDI-TOF mass spectrometry.

Example 12 Measurement of oxidative stress and free radical formation

Prolonged hyperglycemia is linked to the progression of diabetic complications. The mechanisms underlying these processes are not understood, though it is caused by the intracellular production in reactive oxygen species. Without being bound by theory, it is thought that Deglycation Enzyme is the link between prolonged hyperglycemia, resulting in nonenzymatic glycation and free radical production. During the process of enzymatic deglycation, the generation of free radicals by Deglycation Enzyme causes cellular oxidative stress and cellular damage that occurs during prolonged

hyperglycemia. The aim of the experiments in this example and Example 13 is to elucidate the role of Deglycation Enzyme in the production of free radicals and the subsequent effects on cellular damage to prevent induced damage through inhibition of enzymatic activity. Cells (*e.g.*, stably transfected aortic cells) expressing the recombinant Deglycation Enzyme are incubated in low glucose (*e.g.*, 5 mM) or high glucose concentrations (*e.g.*, 30 mM) for several days. Identical experiments are performed in cells transfected with control vector. Effects of prolonged hyperglycemia is confirmed through pathways thought to act via protein kinase C, AGE formation, and the production of sorbitol. These experiments measure whether overexpression of recombinant Deglycation Enzyme sensitizes the cell to damage through increased free radical formation. Superoxide and hydrogen peroxide production are measured by several methods, such as cytochrome c reduction or by commercially available fluorescent probes (*e.g.*, dihydrorhodaminel23 (Molecular Probes).

Example 13 Mutagenesis and deletion of homologous gene in other organisms.

Deglycation Enzyme human sequence is used to isolate and characterize homologous Deglycation Enzyme in other organisms. Some examples are shown in Figure 4. The level of homology in each case is distant with approximately 30-40% sequence identities. A search of sequence data bases reveals the following plants or micro-organisms: *C. elegans, Arabidopsis, Vibrio cholerae, Pasteurella multocida, Neurospora crassa, and E. coli.* Other examples are shown in Figure 5. The other micro-organisms are useful models to explore the biology of the Deglycating Enzyme in glucose metabolism. The corresponding gene in the homologous gene from the other organisms is then deleted from the genome or mutated in a manner that alters function. Deglycation Enzyme is also placed in an expression cassette and used to generate transgenic organisms which overexpress Deglycation Enzyme. An appropriate animal model to study these effects is *Mus musculus*.

Example 14 Deglycation Enzyme variants

Transcriptional or translation variants of Deglycation Enzyme are identified by a sequence search of human genome for homologous or related sequence on chromosome that is essentially identical but lacking the following coding cDNA sequence: tcatgctgca aagcttggag cccagctggc cgatttacac cttgataaca agaagcttgg agagatgcgc ctgaaggagg cgggcacagt g (SEQ ID NO:5)

This sequence corresponds to the following 30 amino acids in the translation:
HAAKLGAQLADLHLDNKKLGEMRLKEAGTV (SEQ ID NO:6)
Transcriptional or translational variants are then tested for functionality, the ability to modulate (*e.g.*, antagonize or agonize) Deglycation Enzyme activity.

Example 15 Binding of Deglycating Enzyme to NEGs

Binding of Deglycating enzyme to NEGs is demonstrated through classical Michaelis Menton enzyme kinetics. Activity of Deglycating Enzyme, is measured in an appropriate assay, for example, by measuring the rate of enzyme-catalyzed products. Activities are then conducted over a range of substrate concentrations and in the presence of fixed concentrations of inhibitors. These inhibitors are either competitive, uncompetitive, or non-competitive antagonists. Analysis of the kinetic data is used to derive parameters for binding of Deglycating enzyme to NEGs. In addition, NEGs are co-crystallized with Deglycating Enzyme to provide direct structural evidence for NEGs binding.

Example 16 Detection of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs

The formation of early glycation products in the glycated proteins and low molecular weight sugar amine is measured by determining fructosamine content using the Fructosamine Test (Hoffmann-La Roche, Switzerland). This test relies on the ability of fructosamine to act as a reducing agent in alkaline solution. Alternatively, fructosamine can be measured using another commercially available fructosamine assay (Polymedco Inc. Catalog No. FRU400). The AGE content of glycated proteins is determined by a noncompetitive and competitive enzyme-linked immunosorbent assay (ELISA) using polyclonal anti-AGE antibody. In addition, method such as HPLC and mass spectrometry are used to measure free low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs.

All patents, publications, scientific articles, web sites, and other documents and materials referenced or mentioned herein are indicative of the levels of skill of those skilled in the art to which the invention pertains, and each such referenced document and material is hereby incorporated by reference to the same extent as if it had been incorporated by reference in its entirety individually or set forth herein in its entirety.

Applicants reserve the right to physically incorporate into this specification any and all materials and information from any such patents, publications, scientific articles, web sites, electronically available information, and other referenced materials or documents.

The specific methods and compositions described herein are representative of preferred embodiments and are exemplary and not intended as limitations on the scope of the invention. Other objects, aspects, and embodiments will occur to those skilled in the art upon consideration of this specification, and are encompassed within the spirit of the invention as defined by the scope of the claims. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, or limitation or limitations, which is not specifically disclosed herein as essential. Thus, for example, in each instance herein, in embodiments or examples of the present invention, any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms in the specification. Also, the terms "comprising", "including", containing", *etc.* are to be read expansively and without limitation. The methods and processes illustratively described herein suitably may be practiced in differing orders of steps, and that they are not necessarily restricted to the orders of steps indicated herein or in the claims. It is also that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus; for example, a reference to "a host cell" includes a plurality (for example, a culture or population) of such host cells, and so forth. Under no circumstances may the patent be interpreted to be limited to the specific examples or embodiments or methods specifically disclosed herein. Under no circumstances may the patent be interpreted to be limited by any statement made by any Examiner or any other official or employee of the Patent and Trademark Office unless such statement is specifically and without qualification or reservation expressly adopted in a responsive writing by Applicants.

The terms and expressions that have been employed are used as terms of description and not of limitation, and there is no intent in the use of such terms and expressions to exclude any equivalent of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of

the invention as claimed. Thus, it will be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

Other embodiments are within the following claims. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

CLAIMS:

1. An isolated polypeptide comprising SEQ ID NO:1.

2. An isolated polypeptide comprising SEQ ID NO:1 or an analog thereof, wherein the polypeptide or said analog is an enzyme.

3. The isolated polypeptide of claim 2, wherein the polypeptide comprises SEQ ID NO:1 with one to five conservatively substituted amino acid residues.

4. The isolated polypeptide of claim 2, wherein the polypeptide comprises SEQ ID NO:1 with an addition or deletion of one to ten amino acid residues.

5. The isolated polypeptide of claim 2, wherein the polypeptide is at least 95% identical to SEQ ID NO:1.

6. The isolated polypeptide of claim 2, wherein the enzyme is capable of modifying a sugar moiety on a polypeptide comprising said sugar moiety.

7. The isolated polypeptide of claim 2, wherein the enzyme is capable of phosphorylating a sugar moiety on a polypeptide comprising said sugar moiety.

8. The isolated enzyme of claim 2 possessing one or more of the following properties:
   a. an ability to bind low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs;
   b. an ability to bind and facilitate the degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs;
   c. ability to bind and facilitate the degradation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs to produce reactive oxygen species; and

d. ability to function as a kinase through phosphorylation of low molecular weight amines and amino acids including fructosamines and other sugar amines or NEGs substrates.

9. The isolated polypeptide of claim 1, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:1 with an amino acid substitution of isoleucine to valine at position 62, an amino acid substitution of arginine to glutamine at position 278, or amino acid substitutions at both positions.

10. An isolated polypeptide comprising a homologue or analog of a polypeptide comprising SEQ ID NO:1, wherein said polypeptide is an antagonist of the non-enzymatic glycation degradation process.

11. A polynucleotide that encodes the isolated polypeptide comprising SEQ ID NO:1.

12. A polynucleotide which encodes the polypeptide according to SEQ ID NO: 1 or a analog thereof.

13. A polynucleotide comprising SEQ ID NO:2.

14. The polynucleotide of claim 13, wherein the polynucleotide encodes a polypeptide comprising SEQ ID NO:1.

15. A polynucleotide which encodes a polypeptide which is homologous to SEQ ID NO: 1 or an analog thereof, wherein the polypeptide binds to non-enzymatic glycation products and antagonizes the catalyzed degradation of one or more of a low molecular weight amine, an amino acid, a sugar amine, or a non-enzymatic glycation product.

16. An expression vector which comprises a polynucleotide which encodes the polypeptide according to SEQ ID NO: 1, or an analog thereof, said

polynucleotide operably linked to an expression control sequence, said expression vector capable of expressing the polypeptide of SEQ ID NO: 1 or said analog.

17. A cultured host cell tranfected with a vector comprising a polynucleotide which encodes for the polypeptide of claim 2, wherein the cell is capable of expressing said polypeptide.

18. A cultured host cell tranfected with a vector comprising a polynucleotide which encodes for the polypeptide according to SEQ ID NO:1, wherein the cell is capable of expressing said polypeptide.

19. A cultured host cell tranfected with a vector comprising a polynucleotide which encodes for a polypeptide that is a homologue or analog of the polypeptide according to SEQ ID NO:1 and which is capable of binding non-enzymatic glycation products and antagonizing the non-enzymatic glycation degradation process, wherein the cell is capable of expressing said polypeptide.

20. A pharmaceutical composition comprising an agent capable of antagonizing the activity of an enzyme having an amino acid sequence according to SEQ ID NO: 1 or an analog thereof.

21. An antibody or antibody fragment that specifically binds a polypeptide comprising SEQ ID NO: 1 or an analog of SEQ ID NO: 1.

22. An antibody of claim 21 that is monoclonal.

23. An antibody of claim 21 or 22 that binds with an affinity of at least about 10^8 M^{-1}.

24. An antibody of claim 21 or 22 that is human or humanized.

25. An isolated cell or a hybridoma capable of secreting the antibody of claim 20.

26. A transcriptional or translational variant comprising an enzyme having an nucleic acid sequence SEQ ID NO: 2 lacking the coding cDNA sequence of SEQ ID NO: 5, wherein said SEQ ID NO: 5 starts at nucleic acid position 311 and stops at position 401 of SEQ ID NO: 2.

27. A method of treating a subject with hyperglycemia comprising administering to said subject a therapeutically effective amount of a polypeptide selected from the group consisting of a polypeptide comprising the amino acid sequence of SEQ. ID NO: 1, a variant of the amino acid sequence of SEQ. ID NO: 1, an analog of the amino acid sequence of SEQ. ID NO: 1, a homolog of the amino acid sequence of SEQ. ID NO: 1, and a bioactive or functional fragment of the amino acid sequence of SEQ. ID NO: 1.

28. A method of treating a subject with diabetes comprising administering to said subject a therapeutically effective amount of a polypeptide selected from the group consisting of a polypeptide comprising the amino acid sequence of SEQ. ID NO: 1, a variant of the amino acid sequence of SEQ. ID NO: 1, an analog of the amino acid sequence of SEQ. ID NO: 1, a homolog of the amino acid sequence of SEQ. ID NO: 1, and a bioactive or functional fragment of the amino acid sequence of SEQ. ID NO: 1.

29. A method of treating a subject with an elevated glycated hemoglobin level comprising administering to said subject a therapeutically effective amount of a polypeptide selected from the group consisting of a polypeptide comprising the amino acid sequence of SEQ. ID NO: 1, a variant of the amino acid sequence of SEQ. ID NO: 1, an analog of the amino acid sequence of SEQ. ID NO: 1, a homolog of the amino acid sequence of SEQ. ID NO: 1, and a bioactive or functional fragment of the amino acid sequence of SEQ. ID NO: 1.

30. The method of claim 29 wherein said glycated hemoglobin level is greater than about 7.0%.

31. A method of treating a subject having or suspected of having an undesired amount of glycated proteins comprising administering to said subject a therapeutically effective amount of a polypeptide selected from the group consisting of a polypeptide comprising the amino acid sequence of SEQ. ID NO: 1, a variant of the amino acid sequence of SEQ. ID NO: 1, an analog of the amino acid sequence of SEQ. ID NO: 1, a homolog of the amino acid sequence of SEQ. ID NO: 1, and a bioactive or functional fragment of the amino acid sequence of SEQ. ID NO: 1.

32. The method of claim 31 wherein said glycated proteins comprise advanced glycation endproducts.

33. The method of claim 31 wherein said glycated proteins comprise non-enzymatic glycation products.

34. A method for screening for agents that modulate the activity of a Deglycating Enzyme, comprising:

a. determining a first amount of low molecular weight sugar moieties or reactive oxygen species or non-enzymatic glycation products in a first biological sample;
b. selecting at least one of said agents for testing;
c. administering at least one agent to said biological sample;
d. determining a second amount of low molecular weight sugar moieties or reactive oxygen species or non-enzymatic glycation products in a said biological sample; and,
e. comparing said first amount with said second amount.

35. A method for screening agents that modulate the activity of a Deglycating Enzyme, comprising:

a. adding an agent or agents to be tested to test medium selected from the group consisting of a cell, cell line, transfected cell line, an isolated tissue sample, and a test animal;
b. determining whether said agent or agents modulate the activity of said Deglycating Enzyme in said test medium.

36. A method for identifying agents that antagonize the activity of a Deglycating Enzyme, comprising:
a. selecting at least one agent for testing;
b. admixing a predetermined amount of said at least one agent with Deglycating Enzyme to form a test mixture; and,
c. determining whether said agent or agents modulate the activity of said Deglycating Enzyme.

37. A method for screening for compounds that antagonize a Deglycating Enzyme activity, comprising monitoring Deglycating Enzyme activity following administration of one or more of said compounds to a test medium.

38. A method for determining the ability of an agent to modulate the actitivty of a Deglycating Enzyme activity in a test medium, comprising:
a. exposing said test medium to said agent;
b. determining a first test value, comprising at least one of the pre-exposure rate of degradation of low molecular weight amines and amino acids including non-enzymatic glycation products, the pre-exposure rate of generation of reactive oxygen species, the pre-exposure amount of low molecular weight amines and amino acids including non-enzymatic glycation products, the pre-exposure amount of free low molecular weight amines and amino acids including non-enzymatic glycation products, and the pre-exposure amount of reactive oxygen species said test medium;
c. determining a second test value, comprising at least one of the post-exposure rate of degradation of low molecular weight amines and amino acids including non-enzymatic glycation products, the

post-exposure rate of generation of reactive oxygen species, the post-exposure amount of low molecular weight amines and amino acids including non-enzymatic glycation products, the post-exposure amount of free low molecular weight amines and amino acids including non-enzymatic glycation products, and the post-exposure amount of reactive oxygen species in said test medium; and

d. comparing said first test value with said second test value.

39. A method for providing treatment to an individual, comprising administering to said individual an effective amount of an agent that antagonizes the activity of a Deglycating Enzyme.

40. The method of claim 39, wherein said individual has one or more complications associated with diabetes.

41. The method of claim 39, wherein said individual has a condition that results in or involves an undesired amount of non-enzymatic glycation products.

42. A method for providing prophylactic treatment to an individual, comprising administering to said individual a prophylactic effective amount of an agent that antagonizes a Deglycating Enzyme.

43. The method of claim 42, wherein said treatment is provided in order to ameliorate or delay the development of one or more complications associated with diabetes.

44. The method of claim 42, including wherein said treatment ameliorates or delays development of a condition associated with an undesired amount of degradation of non-enzymatic glycation products.

45. A method for providing treatment to an individual, comprising administering to said individual an effective amount of a Deglycating Enzyme.

46. The method of claim 45, wherein said individual has one or more complications associated with diabetes.

47. The method of claim 45, wherein said individual has a condition that results in or involves an undesired amount of non-enzymatic glycation products.

48. A method for providing prophylactic treatment to an individual, comprising administering to said individual a prophylactic effective amount of a Deglycating Enzyme.

49. The method of claim 48, wherein said treatment is provided in order to ameliorate or delay the development of one or more complications associated with diabetes.

50. The method of claim 48, including wherein said treatment ameliorates or delays development of a condition associated with an undesired amount of degradation of non-enzymatic glycation products.

51. A method for modulating degradation of non-enzymatic glycation products in an individual, comprising administering to said individual an effective amount of an agent comprising a Deglycating Enzyme.

52. A method for modulating degradation of non-enzymatic glycation products in an individual, comprising administering to said individual an effective amount of an agent comprising a Deglycating Enzyme.

53. A method for preparing an antibody that binds a Deglycating Enzyme comprising:

a. inoculating a test animal with a Deglycating Enzyme or a Deglycating Enzyme analog;
b. obtaining lymphocytes from said test animal;
c. fusing said lymphocytes with a myeloma cell line to produce a hybridoma;
d. culturing said hybridoma to produce monoclonal antibodies; and

e. selecting from said monoclonal antibodies those monoclonal antibodies that bind to said Deglycating Enzyme or said Deglycating Enzyme analog.

54. A method for identifying substances that interact with Deglycating Enzyme, comprising:
    a. admixing a Deglycating Enzyme with a biological sample; and
    b. detecting the interaction of said Deglycating Enzyme with at least one component of said biological sample.

55. The method of claim 54, wherein said substances are selected from the group consisting of proteins and nucleic acids.

56. A method for identifying variants of a Deglycating Enzyme comprising probing an animal genome using a portion of a Deglycating Enzyme.

57. The method of claim 56, wherein said variants are transcriptional variants.

58. The method of claim 56, wherein said variants are translational variants.

59. An antibody that binds to a polypeptide having the sequence of SEQ ID NO. 1.

60. The antibody of claim 59 wherein said antibody is a polyclonal antibody.

61. The antibody of claim 59 wherein said antibody is a monoclonal antibody.

62. The antibody of claim 59 wherein said antibody comprises an antibody Fv fragment.

Figure 1


4072 bp
3054 bp
2036 bp
1636 bp
1018 bp
506 bp
396 bp
2996 bp Band
556 bp Band
376 bp Band

Figure 2

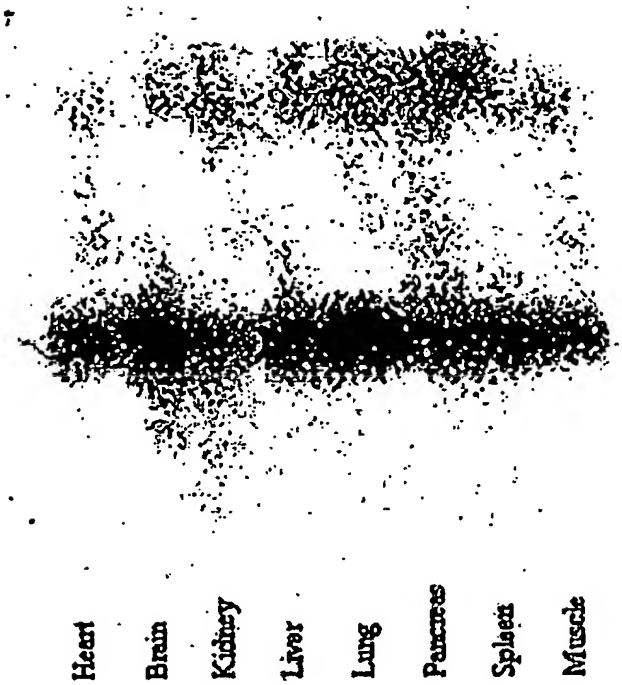

Figure 3

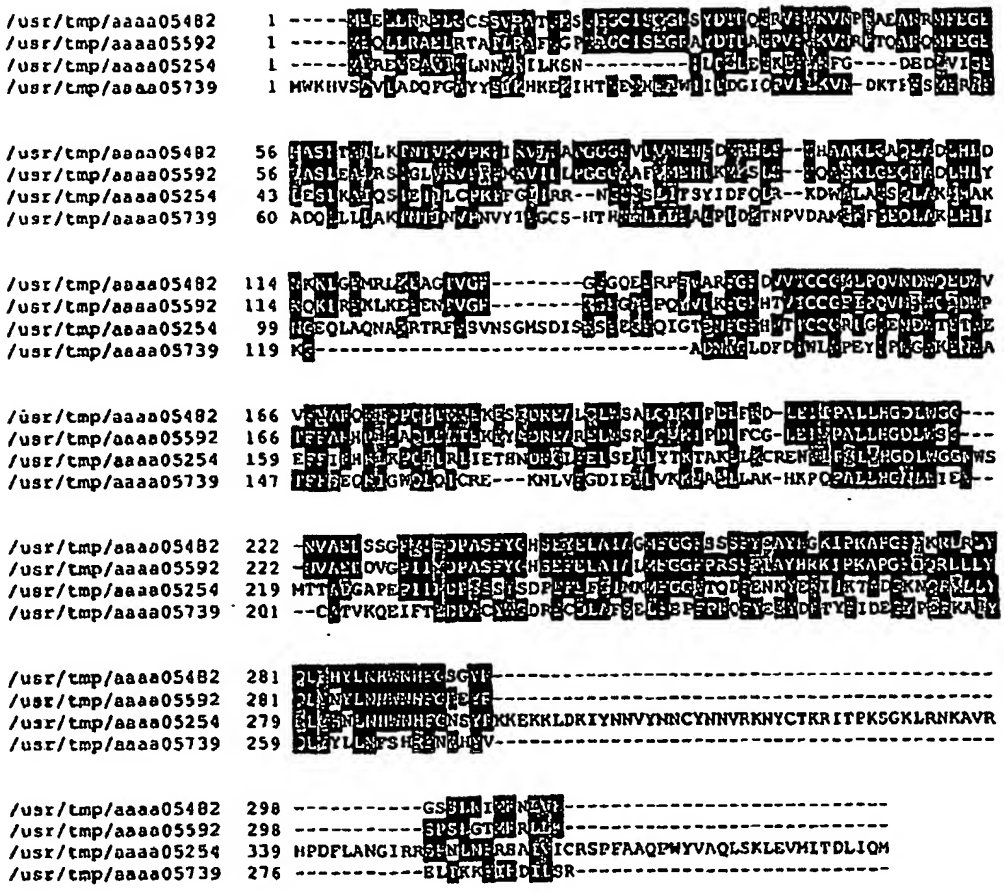

Figure 4

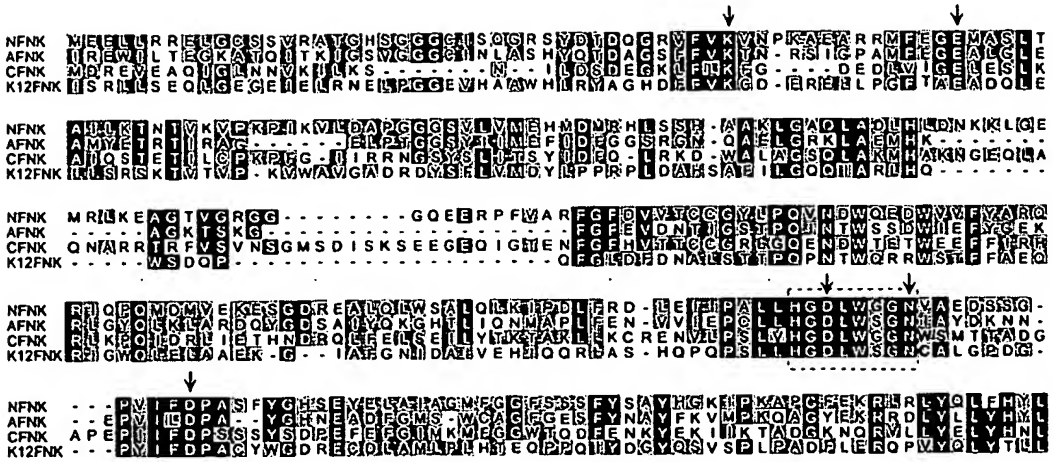

Figure 5

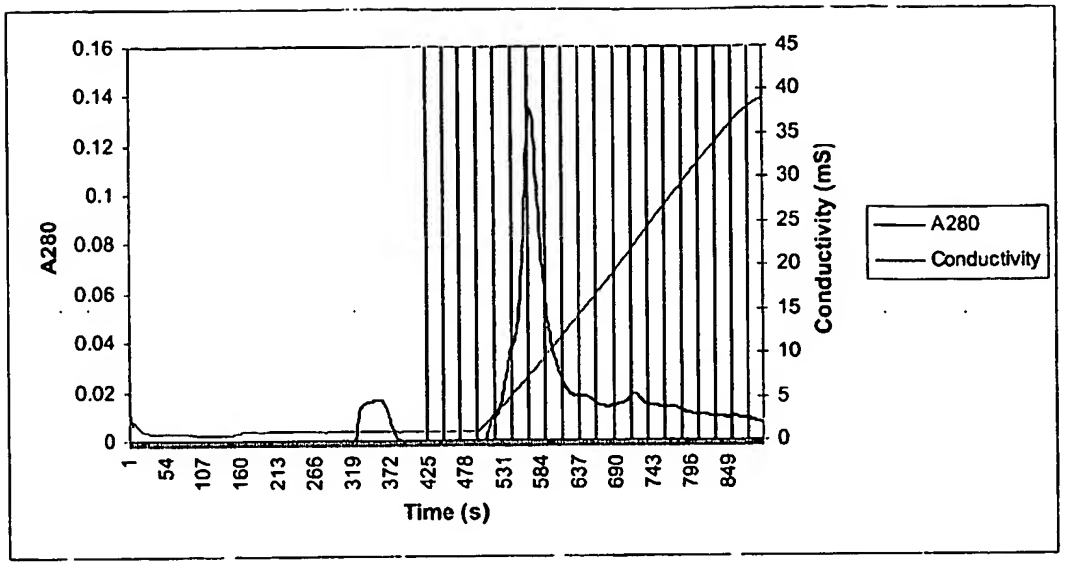
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
A280
45
40
35
30
25
20
15
10
5
0
Conductivity (mS)
1
54
107
160
213
266
319
372
425
478
531
584
637
690
743
796
849
Time (s)
A280
Conductivity

Figure 6

Figure 7

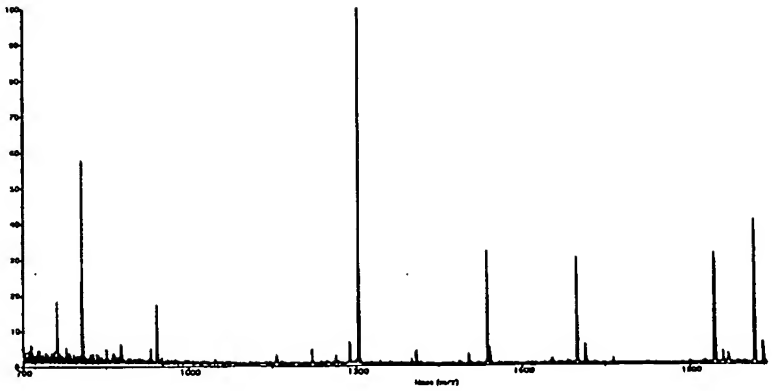

Figure 8

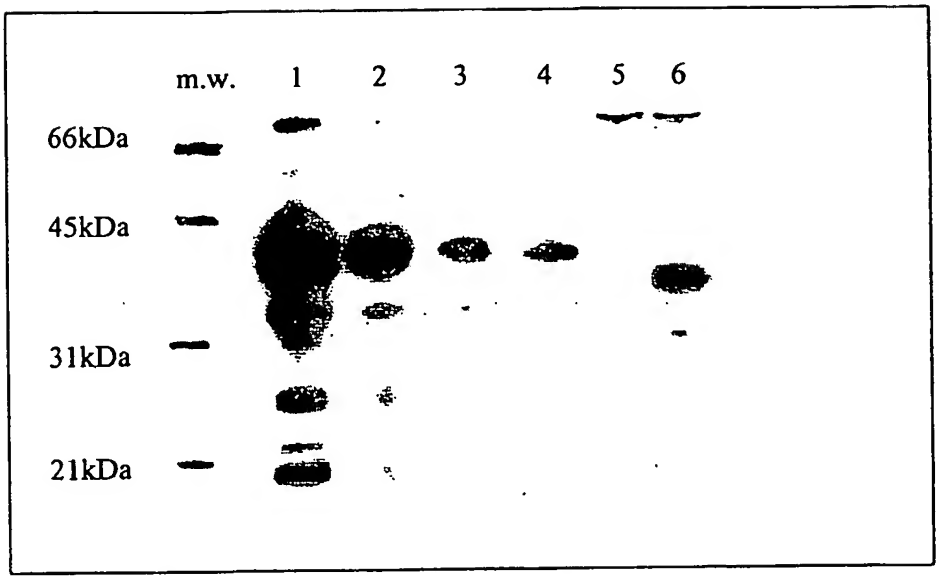

Figure 9

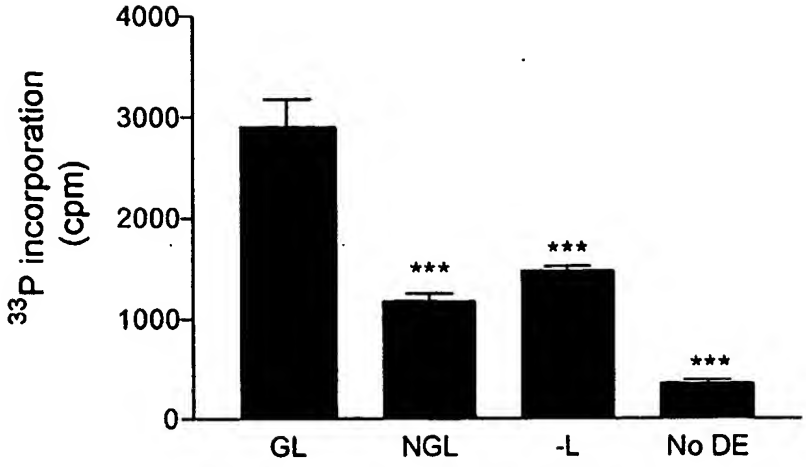

Figure 10

SEQUENCE LISTING

<110> Protemix Corporation Limited
Cooper, Garth
Loomes, Kerry

<120> Proteins with Deglycating Activities and Methods of Using the Same

<130> 49123-000007/481188

<150> US 60/376,926
<151> 2002-04-29

<150> NZ 524892
<151> 2003-03-21

<160> 18

<170> PatentIn version 3.2

<210> 1
<211> 309
<212> PRT
<213> Homo sapiens

<400> 1

Met Glu Glu Leu Leu Arg Arg Glu Leu Gly Cys Ser Ser Val Arg Ala
1 5 10 15

Thr Gly His Ser Gly Gly Gly Cys Ile Ser Gln Gly Arg Ser Tyr Asp
20 25 30

Thr Asp Gln Gly Arg Val Phe Val Lys Val Asn Pro Lys Ala Glu Ala
35 40 45

Arg Arg Met Phe Glu Gly Glu Met Ala Ser Leu Thr Ala Ile Leu Lys
50 55 60

Thr Asn Thr Val Lys Val Pro Lys Pro Ile Lys Val Leu Asp Ala Pro
65 70 75 80

Gly Gly Gly Ser Val Leu Val Met Glu His Met Asp Met Arg His Leu
85 90 95

Ser Ser His Ala Ala Lys Leu Gly Ala Gln Leu Ala Asp Leu His Leu
100 105 110

Asp Asn Lys Lys Leu Gly Glu Met Arg Leu Lys Glu Ala Gly Thr Val
115 120 125

Gly Arg Gly Gly Gly Gln Glu Glu Arg Pro Phe Val Ala Arg Phe Gly
130 135 140

Phe Asp Val Val Thr Cys Cys Gly Tyr Leu Pro Gln Val Asn Asp Trp
145 150 155 160

Gln Glu Asp Trp Val Val Phe Tyr Ala Arg Gln Arg Ile Gln Pro Gln

165 170 175

Met Asp Met Val Glu Lys Glu Ser Gly Asp Arg Glu Ala Leu Gln Leu
180 185 190

Trp Ser Ala Leu Gln Leu Lys Ile Pro Asp Leu Phe Arg Asp Leu Glu
195 200 205

Ile Ile Pro Ala Leu Leu His Gly Asp Leu Trp Gly Gly Asn Val Ala
210 215 220

Glu Asp Ser Ser Gly Pro Val Ile Phe Asp Pro Ala Ser Phe Tyr Gly
225 230 235 240

His Ser Glu Tyr Glu Leu Ala Ile Ala Gly Met Phe Gly Gly Phe Ser
245 250 255

Ser Ser Phe Tyr Ser Ala Tyr His Gly Lys Ile Pro Lys Ala Pro Gly
260 265 270

Phe Glu Lys Arg Leu Arg Leu Tyr Gln Leu Phe His Tyr Leu Asn His
275 280 285

Trp Asn His Phe Gly Ser Gly Tyr Arg Gly Ser Ser Leu Asn Ile Met
290 295 300

Arg Asn Leu Val Lys
305

<210> 2
<211> 1678
<212> DNA
<213> Homo sapiens

<400> 2
gcggccgcgg cgggaacatg gaggagctgc tgaggcgcga gctgggctgc agctctgtca 60

gggccacggg ccactcgggg ggcgggtgca tcagccaggg ccggagctac gacacggatc 120

aaggacgagt gttcgtgaaa gtgaacccca aggcggaggc cagaagaatg tttgaaggtg 180

agatggcaag tttaactgcc atcctgaaaa caaacacggt gaaagtgccc aagcccatca 240

aggttctgga tgccccaggc ggcgggagcg tgctggtgat ggagcacatg gacatgaggc 300

atctgagcag tcatgctgca aagcttggag cccagctggc cgatttacac cttgataaca 360

agaagcttgg agagatgcgc ctgaaggagg cgggcacagt ggggagagga ggtgggcagg 420

aggaacggcc ctttgtggcc cggtttggat ttgacgtggt gacgtgctgt ggatacctcc 480

cccaggtgaa tgactggcag gaggactggg tcgtgttcta tgcccggcag cgcattcagc 540

cccagatgga catggtggag aaggagtctg gggacaggga ggccctccag ctttggtctg 600

ctctgcagtt aaagatccct gacctgttcc gtgacctgga gatcatccca gccttactcc 660

acggggacct ctggggtgga aacgtagcag aggattcctc tgggccggtg atttttgacc 720

cagcttcttt ctacggccac tcggaatatg agctggcaat agctggcatg tttgggggct 780

ttagcagctc cttttactcc gcctaccacg gcaaaatccc caaggcccca ggattcgaga 840

agcgccttcg gttgtatcag ctctttcact acttgaacca ctggaatcat tttggatcgg 900

ggtacagagg atcctccctg aacatcatga ggaatctggt caagtgagcg ggccttactc 960

tggaaggagg cctcagaggt ttctccacag tcctcttctg ggcaaattct tgtttcttca 1020

catgccggac tagcttaaga ccaatgcagt agcttatttc caagccttgc aaagtatata 1080

atatctaaga ggaaaggttt tgtcatccca gcgttgtcca ctttgtgggg ctttgtaggt 1140

agacggagcc acactacagg cagggtatga gcagagggat gtatggagtg tgggtgactc 1200

tgagcctcac tgctgctgca aggtggggaa actgtaagtg aacccctgtg ggtgcggggg 1260

agggtatccg gtgcgcaggg aggtggccag cgcccccggg cactgctgct cataggtacc 1320

tttccactgc ctcctccctg ctctcctgtg caggaatgtc tctgagctgt tcacgttgat 1380

gcttcttggt tggcaagact tgggtgtaga catgaaacca tcttactaaa agtgtcttaa 1440

aatgaccaat tccagaatca agcgtattcc gttttcttcc tgcatgatcc ctgggccctc 1500

ccgcaggctg agcaagtctg taaactgatt ctgggagaaa ccaagctgct ggccataggg 1560

tgtccttggg tacatccagg agtcttcatt gcttctgtta ttaccccgtc tcctctgcca 1620

ttttctacag cttgctgagt tgtcattcct ttgcaacatt aaaatacatg ctgaactc 1678

<210> 3
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Obtained Commercially

<400> 3
atggaggagc tgctgagg 18

<210> 4
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Obtained Commercially

<400> 4
tcacttgacc agattcctca tgatgtt 27

<210> 5
<211> 91
<212> DNA
<213> Artificial Sequence

<220>
<223> Portion of Declycation Enzyme Nucleic Acid Sequence

<400> 5
tcatgctgca aagcttggag cccagctggc cgatttacac cttgataaca agaagcttgg 60

agagatgcgc ctgaaggagg cgggcacagt g 91

<210> 6
<211> 30
<212> PRT
<213> Artificial Sequence

<220>
<223> Portion of Deglycation Enzyme Amino Acid Sequence

<400> 6

His Ala Ala Lys Leu Gly Ala Gln Leu Ala Asp Leu His Leu Asp Asn
1 5 10 15

Lys Lys Leu Gly Glu Met Arg Leu Lys Glu Ala Gly Thr Val
20 25 30

<210> 7
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Test Sequence

<400> 7

Ile Pro Asp Leu Phe Arg
1 5

<210> 8
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> Test Sequence

<400> 8

Ala Pro Gly Phe Glu Lys Arg
1 5

<210> 9
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Test Sequence

<400> 9

Gly Ser Ser Leu Asn Ile Met Arg
1 5

<210> 10
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Test Sequence

<400> 10

Ser Tyr Asp Thr Asp Gln Gly Arg
1 5

<210> 11
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> Test Sequence

<400> 11

Gly Gly Gly Gln Glu Glu Arg Pro Phe Val Ala Arg
1 5 10

<210> 12
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Test Sequence

<400> 12

Leu Gly Ala Gln Leu Ala Asp Leu His Leu Asp Asn Lys
1 5 10

<210> 13
<211> 14
<212> PRT
<213> Artificial Sequence

<220>
<223> Test Sequence

<400> 13

Leu Gly Ala Gln Leu Ala Asp Leu His Leu Asp Asn Lys Lys
1 5 10

<210> 14
<211> 14
<212> PRT
<213> Artificial Sequence

<220>
<223> Test Sequence

<400> 14

Met Phe Glu Gly Glu Met Ala Ser Leu Thr Ala Ile Leu Lys
1 5 10

<210> 15
<211> 15
<212> PRT
<213> Artificial Sequence

<220>

<223> Test Sequence

<400> 15

Arg Met Phe Glu Gly Glu Met Ala Ser Leu Thr Ala Ile Leu Lys
1 5 10 15

<210> 16
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> Test Sequence

<400> 16

Glu Ser Gly Asp Arg Glu Ala Leu Gln Leu Trp Ser Ala Leu Gln Leu
1 5 10 15

Lys

<210> 17
<211> 19
<212> PRT
<213> Artificial Sequence

<220>
<223> Test Sequence

<400> 17

Val Leu Asp Ala Pro Gly Gly Gly Ser Val Leu Val Met Glu His Met
1 5 10 15

Asp Met Arg

<210> 18
<211> 8
<212> PRT
<213> Multiple Organisms

<400> 18

His Gly Asp Leu Trp Gly Gly Asn
1 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ03/00074

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C07K 14/47; C12N 9/12; A61K 38/45; A61P 3/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Gen Pept., RefSeq, PDB, Swiss Prot., PIR, PRF, EMBL: SEQ ID Nos 1 and 2;
STN File Medline, CA, WPIDS, Biosis Keywords: deglycat?, glycat?, enym?, fructosamine kinase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database Swiss Prot Accession Number Q9HA64, Q9H0U7, submitted August 2000 & WIEMANN, S et al "Toward a catalog of human genes and proteins: sequencing and analysis of 500 novel complete protein coding human cDNAs". Genome Res. (2001) vol 11 pages 422-435. See Swiss Prot abstract	1-26, 34-38, 53-62
X	Database GenPept Accession Number AAH07611, submitted 10 May 2001	1-26, 34-38, 53-62
X	Database EMBL Accession Number AK022233, submitted 23 August 2000 (See also Swiss Prot Q9HA64)	13-19, 26 and 56-58

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
26 June 2003

Date of mailing of the international search report
03 JUL 2003

Name and mailing address of the ISA/AU

AUSTRALIAN PATENT OFFICE
PO BOX 200, WODEN ACT 2606, AUSTRALIA
E-mail address: pct@ipaustralia.gov.au
Facsimile No. (02) 6285 3929

Authorized officer

CHRISTINE BREMERS
Telephone No : (02) 6283 2313

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ03/00074

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database EMBL Accession Number HSM801604, submitted 18 January 2000 (See also Swiss Prot Q9HA64)	13-19, 26 and 56-58
X	Database Genbank Accession Number BC014408, submitted 17 September 2001	13-19, 26 and 56-58
X	Database EMBL Accession Number BC007611, submitted 10 May 2001	13-19, 26 and 56-58
X	DELPIERRE, G et al "Fructosamine 3-kinase is involved in an intracellular deglycation pathway in human erythrocytes". Biochem. J. (25 April 2002) vol 365 pages 801-808 See abstract, page 801, page 803, page 806	45-58
X	BULTEAU, A-L et al "Proteasome inhibition in glyoxal-treated fibroblasts and resistance of glycated glucose-6-phosphate dehydrogenase to 20 S proteasome degradation in vitro". The Journal of Biological Chemistry (2001) vol 276 no 49 pages 45662-45668 See abstract	45-58
X	SWERGOLD, B S et al "Purification, sequencing, substrate specificity, and evidence of activity in vivo". Diabetes (2001) vol 50 no 9 pages 2139-2147 See abstract, page 2139- page 2140 second paragraph, page 2145 column 2, page 2146 column 1	39-58
X	STN File Medline abstract no 2002178536 & Diabetes Technol Ther, (2001) vol 3 no 4 pages 609-616 See abstract	45-58
X	TAKAHASHI, M et al "Molecular cloning and expression of amadoriase isoenzyme (fructosyl amine:oxygen oxidoreductase, EC 1.5.3) from Aspergillus fumigatus. The Journal of Biological Chemistry (1997) vol 272 no 19 pages 12505-12507 See page 12505 the abstract and column 1 first paragraph, column 2 first paragraph	39-44, 45-58

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ03/00074

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TAKAHASHI, M et al "Isolation, purification, and characterization of amadoriase isoenzymes (fructosyl amine-oxygen oxidoreductase EC 1.5.3) from Aspergillus sp. The Journal of Biological Chemistry (1997) vol 272 no 6 pages 3437-3443 See page 3437 column 2 second paragraph, page 3441 column 2 fourth paragraph, page 3442, Table IV	45-58
X	SAXENA, A K et al "Purification and Characterization of a membrane-bound deglycating enzyme (1-deoxyfructosyl alkyl amino acid oxidase, EC 1.5.3) from a Pseudomonas sp. soil strain". The Journal of Biological Chemistry (1996) vol 271 no 51 pages 32803-32809 See page 32803	45-58